METHODS OF TREATING MULTIPLE
MYELOMA AND MYELOMA-INDUCED BONE
RESORPTION USING INTEGRIN ANTAGONISTS

This utility application is a continuation-in-
5   part of United States application number 09/943,659, filed
August 31, 2001, which is a continuation-in-part of United
States application number 09/805,840, filed March 13,
2001, which is a continuation of PCT application number
PCT/US99/21170, filed September 13, 1999, which claims
10 benefit of United States provisional application number
60/100,182, filed September 14, 1998. The disclosures of
United States application numbers 09/943,659 and
09/805,840, PCT application number PCT/US99/21170 and
United States provisional application number 60/100,182
15 are incorporated herein by reference.

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a treatment for
multiple myeloma, and the release of bone-resorting
factors by myeloma cells, resulting in severe bone loss,
20 which is the major side-effect of myeloma in man. More
particularly, this invention relates to integrin
antagonists, such as antagonists of alpha4 containing

integrins, which inhibit the biological effects of such
adhesion, associated with homing of multiple myeloma cells
to bone marrow; their subsequent integrin- dependent
survival; and their integrin-dependent release of bone-
5   resorbing factors, resulting in bone destruction in
patients with multiple myeloma.

BACKGROUND OF THE INVENTION

Multiple myeloma is a B-cell malignancy that has
strong predilection for colonizing the bone marrow and is
10   associated with severe osteoclastic bone resorption.
Multiple myeloma is the second most common hematologic
malignancy, with 15,000 new cases diagnosed each year and
30,000 to 40,000 myeloma patients in the U.S. annually
(Mundy and Bertolini 1986). Eighty percent of the
15   patients suffer from devastating osteolytic bone
destruction caused by increased osteoclast (OCL) formation
and activity (Mundy and Bertolini 1986). This bone
destruction can cause excruciating bone pain, pathologic
fractures, spinal cord compression, and life-threatening
20   hypercalcemia. Because multiple myeloma cannot be cured
by standard chemotherapy or stem cell transplantation
(Attal et al., 1996), and because of the severe morbidity
and potential mortality associated with myeloma bone
disease, treatment strategies that control the myeloma
25   growth itself, and in particular the osteolytic bone
destruction that occurs in these patients, are vitally
important.

However, the pathologic mechanisms responsible
for the increased osteoclast activity in patients with
30   multiple myeloma are unknown (Mundy, 1998). The bone

lesions occur in several patterns. Occasionally, patients
develop discrete osteolytic lesions that are associated
with solitary plasmacytomas. Some patients have diffuse
osteopenia, which mimics the appearance of osteoporosis,
5   and is due to the myeloma cells being spread diffusely
throughout the axial skeleton. In most patients there are
multiple discrete lytic lesions occurring adjacent to
nests of myeloma cells. Hypercalcemia occurs as a
consequence of bone destruction in about one-third of
10   patients with advanced disease. Rarely, patients with
myeloma do not have lytic lesions or bone loss, but rather
have an increase in the formation of new bone around
myeloma cells. This rare situation is known as
osteosclerotic myeloma.
15   Osteolytic bone lesions are by far the most
common skeletal manifestations in patients with myeloma
(Mundy, 1998). Although the precise molecular mechanisms
remain unclear, observations over 15 years have shown
that: 1) The mechanism by which bone is destroyed in
20   myeloma is via the osteoclast, the normal bone-resorbing
cell; 2) Osteoclasts accumulate on bone-resorbing surfaces
in myeloma adjacent to collections of myeloma cells and it
appears that the mechanism by which osteoclasts are
stimulated in myeloma is a local one; 3) It has been known
25   for many years that cultures of human myeloma cells in
vitro produce several osteoclast activating factors;
including lymphotoxin-alpha (LT-α), interleukin-1(IL-1),
parathyroid-hormone related protein (PTHrP) and
interleukin-6 (IL-6); 4) Hypercalcemia occurs in
30   approximately one-third of patients with myeloma some time
during the course of the disease. Hypercalcemia is always

associated with markedly increased bone resorption and frequently with impairment in glomerular filtration;

5) The increase in osteoclastic bone resorption in myeloma is usually associated with a marked impairment in

5 osteoblast function. Alkaline phosphatase activity in the serum is decreased or in the normal range, unlike patients with other types of osteolytic bone disease, and radionuclide scans do not show evidence of increased uptake, indicating impaired osteoblast responses to the

10 increase in bone resorption.

Although various mediators listed above have been implicated in the stimulation of osteoclast activity in patients with multiple myeloma, reports of factors produced by myeloma cells have not been consistent, and

15 some studies have been inconclusive due to the presence of other contaminating cell types, including stromal cells and macrophages, in the multiple myeloma cell population. IL-6 is a major myeloma growth factor that enhances the growth of several myeloma cell lines and freshly isolated

20 myeloma cells from patients (Bataille et al., 1989). IL-6 production can be detected in about 40% of freshly isolated myeloma cells by PCR, but only 1 in 150 patients studied show detectable IL-6 production by immunocytochemistry or ELISA assays (Epstein 1992). The

25 IL-6 receptors were only detected in 6 of 13 samples from patients with multiple myeloma (Bataille et al., 1992). Furthermore, mature myeloma cells have been reported to have a minimal proliferative response to IL-6. Interleukin-11 (IL-11) has an IL-6-like activity on

30 plasmacytomas, but to date no one has demonstrated that myeloma cells produce IL-11. Bataille and coworkers

(1995) have shown that perfusion of 5 patients with refractory myeloma with an antibody to IL-6 decreased the size of the myeloma cell burden in only 2 of these patients. IL-1 is an extremely potent bone resorbing agent that induces hypercalcemia in animal models in the absence of renal failure (Boyce et al., 1989). In contrast, hypercalcemia rarely occurs in myeloma patients without renal failure. More importantly, in highly purified myeloma cells, no IL-1 and only rare TNF-α production can be detected, suggesting that other contaminating cell types such as macrophages may be the source of IL-1 and TNF-α (Epstein 1992). Similarly, LT-α is produced by most human myeloma cell lines (Bataille et al., 1995) but does not appear to be produced by myeloma cells in vivo (Alsina et al., 1996). In addition to IL-1, TNF-α, LT-α, and IL-6, myeloma cells produce a truncated form of M-CSF which is biologically active, but M-CSF does not cause hypercalcemia or induce osteoclast formation by itself in human marrow cultures (MacDonald et al., 1986).

Thus, the role of any of these factors in osteolytic bone disease in patients with myeloma has not been clearly demonstrated in vivo, so that known cytokines clearly do not totally account for the bone resorption seen in these patients.

Role of Adhesive Molecule
Interactions in Myeloma Bone Disease

Anderson and coworkers were the first group to demonstrate the importance of adhesive interactions between myeloma cells and cells in the marrow

microenvironment both in the growth of myeloma cells and
the development of osteolytic bone disease. Multiple
myeloma cells express cell surface adhesion molecules,
CD29 (VLA-4), LFA-1, and CD44 (Chauhan et al., 1995).
5 These workers suggested that myeloma cells localized to
the marrow via specific adhesion interactions between
extracellular matrix proteins and bone marrow stromal
cells. They further showed that adhesion of multiple
myeloma cells to stromal cells triggered IL-6 secretion by
10 both normal and multiple myeloma bone marrow-derived
stromal cells and increased IL-6-mediated tumor cell
growth. However, antibodies to CD29, LFA-1 or CD44 did
not decrease IL-6 production by marrow stromal cells in
response to myeloma cells, suggesting that another ligand-
15 receptor interaction triggered the IL-6 secretion by bone
marrow stromal cells binding to myeloma cells. Mere
identification of a possible adhesion pathway does not
necessarily mean that the pathway is important. In this
case none of the implicated pathways plays a role in IL-6
20 production.

Vanderkerken et al. (1997) also examined the
phenotypic adhesion profile of murine 5T2 cells and ST33
myeloma cells in a model of murine myeloma. These
investigators showed that these cell lines expressed
25 VLA-4, VLA-5, LFA-1, and CD44, and suggested that these
adhesive interactions might be important for myeloma cells
to bind to marrow stromal cells.

Nevertheless, despite many laboratory advances,
the fundamental mechanisms underlying increased
30 osteoclastic bone destruction in myeloma in vivo remain
poorly understood. This is reflected in the inability to

easily translate the data on adhesive interactions
obtained in vitro to the in vivo setting. For example,
many in vitro studies implicate both the integrin VLA-4
and the integrin LFA-1 in the adhesion of hematopoietic

5   stem cells to bone marrow stroma (reviewed in
Papayannopoulou and Nakamoto, 1993). These in vitro data
would predict that either pathway, if blocked in vivo,
would result in peripheralization of hematopoietic stem
cells from marrow to peripheral blood. Yet, in a primate

10   study, while a monoclonal antibody (mAb) to VLA-4
effectively peripheralized stem cells, a monoclonal
antibody to the beta2 integrin chain of LFA-1 was without
effect, despite increasing neutrophil counts, thus
demonstrating the efficacy of the mAb (Papayannopoulou and

15   Nakamoto, 1993). These data show that the in vitro
results were in fact unable to accurately predict in vivo
relevance.

It should be noted that the role of integrin
VLA-4 has been studied in metastasis of multiple tumors,

20   including leukemias such as lymphoma, with contradictory
results. Thus, transfection of the human alpha 4 chain
into Chinese Hamster Ovary (CHO) cells resulted in VLA-4
expression, and rendered these cells able to migrate to
bone marrow in vivo, a phenomenon inhibited by mAbs to

25   VLA-4 (Matsuura et al., 1996). In contrast, transfection
of lymphoma cells with VLA-4 strongly inhibited metastasis
to liver, lung and kidney, and was without effect on
homing and proliferation in marrow (Gosslar et al., 1996).
In addition, expression of VLA-4 on highly metastatic

30   murine melanoma cells strongly inhibited the formation of

pulmonary metastases in vivo (Qian et al., 1994), and did not predispose melanoma to bone marrow metastasis.

In summary it is not clear on the basis of in vitro studies, how to reliably predict in vivo relevance of adhesion pathways. Furthermore, even when in vivo studies have been performed, the resultant data are inconsistent. One major reason for the perplexing inconsistencies in the field of multiple myeloma is that currently available animal models are not good predictors of human disease. In the case of multiple myeloma, human and murine myeloma cell lines which can be grown in vitro rarely are associated with bone destruction in vivo (Mundy 1998).

It would be highly desirable to identify compounds or antagonists which inhibit production of these bone-resorbing factors, thus halting progressive bone destruction and improving the quality of life of patients with myeloma.

SUMMARY OF THE INVENTION

We have used a recently developed murine model of multiple myeloma in which the mouse develops severe osteolysis with all the hallmarks of human disease (Garrett 1997). Using this cell line and animal model we have established that inhibition of the $\alpha4$ integrin/$\alpha4$ integrin ligand pathway in vivo leads to reduced capacity for multiple myeloma cells to proliferate and/or survive. We show that cell-cell attachment between myeloma cells and marrow stromal cells via the VLA-4/VCAM-1 interaction is required for an increase in the production of an

activity which stimulates osteoclastic bone resorption in the bone microenvironment in vitro.

We propose that this interaction is critical to the homing of myeloma cells to the marrow compartment, to

5 their subsequent survival and growth, to ultimately to the progression of myeloma-induced osteolysis. We tested this in the animal model and found that, in vivo, an antagonist of the alpha4 subunit-containing integrin VLA-4 strongly inhibits the production of antibody of the IgG2b subtype.

10 This isotype is the same as that produced by the 5TGM1 cell line, and is an accurate surrogate for the number of myeloma cells in the marrow compartment at any time. Thus, blockade of the VLA-4 pathway strongly inhibits IgG2b production, and by implication, the level of myeloma

15 burden.

One aspect of the invention is a method for treating multiple myeloma comprising administering to an individual a therapeutically effective amount of a composition comprising an antagonist of an interaction

20 between an integrin with an $\alpha 4$ subunit (e.g., VLA-4) and a ligand for this integrin (e.g., VCAM-1). This antagonist can be an $\alpha 4$ integrin binding agent or an $\alpha 4$ integrin ligand binding agent. Preferred agents are anti-VLA4 or anti-$\alpha 4\beta 7$ antibody homologs (human antibody, a

25 chimeric antibody, a humanized antibody and fragments thereof); anti-VCAM-1 antibody homologs (a human antibody; a chimeric antibody, a humanized antibody and fragments thereof); and a small molecule inhibitor of interactions of $\alpha 4$ subunit containing integrins with their ligands.

30 The composition can be administered at a dosage so as to provide from about 0.1. to about 20 mg/kg body weight. In

particular, the preferred agents can antagonize an interaction: a) of both VLA-4 and $\alpha 4\beta 7$ collectively with their respective $\alpha 4$ ligands; or b) only of VLA-4 with its $\alpha 4$ ligand; or c) only of $\alpha 4\beta 7$ with its $\alpha 4$ ligand. One or

5 more antagonists of an interaction between an integrin with an $\alpha 4$ subunit and a ligand for this integrin could be administered in combination with one or more compounds that preferably are not an antagonist of an interaction between an integrin with an $\alpha 4$ subunit and a ligand for

10 this integrin. Preferably, the compound to be administered in combination with an antagonist of an interaction between an integrin with an $\alpha 4$ subunit and a ligand for this integrin is a chemotherapeutic agent. Preferably, that chemotherapeutic agent is melphalan.

15   Another aspect of the invention is a method for inhibiting bone resorption associated with tumors of bone marrow, the method comprising administering to a mammal with said tumors an antagonist of an interaction between an $\alpha 4$ subunit containing integrin such as VLA-4 and a

20 ligand for this $\alpha 4$ subunit containing integrin, such as VCAM-1, in an amount effective to provide inhibition of the bone resorption. This antagonist can be an $\alpha 4$ integrin binding agent such as a VLA-4 binding agent or an $\alpha 4$ integrin ligand binding agent such as a VCAM-1 binding

25 agent. Preferred agents are anti-VLA4 or and $\alpha 4\beta 7$ antibody homologs (human antibody, a chimeric antibody, a humanized antibody and fragments thereof); anti-VCAM-1 antibody homologs (a human antibody, a chimeric antibody, a humanized antibody and fragments thereof); and a small

30 molecule inhibitor of the interaction of $\alpha 4$ subunit-containing integrins with their respective $\alpha 4$ integrin

ligands (e.g, the VCAM-1/VLA-4 interaction). The
antagonist can be administered at a dosage so as to
provide from about 0.1 to about 20 mg/kg body weight.
One or more antagonists of an interaction between an
5   integrin with an $\alpha 4$ subunit and a ligand for this
integrin could be administered in combination with one or
more compounds that preferably are not an antagonist of an
interaction between an integrin with an $\alpha 4$ subunit and a
ligand for this integrin. Preferably, the compound to be
10 administered in combination with an antagonist of an
interaction between an integrin with an $\alpha 4$ subunit and a
ligand for this integrin is a chemotherapeutic agent.
Preferably, that chemotherapeutic agent is melphalan.

Yet another aspect of the invention is a method
15 of treating a subject having a disorder characterized by
the presence of osteoclastogenesis, the method comprising
administering to the subject an antagonist of an
interaction between an $\alpha 4$ subunit bearing integrin and a
ligand for an $\alpha 4$ subunit-bearing integrin, in an amount
20 sufficient to suppress the osteoclastogenesis. Similarly,
the antagonist can be an $\alpha 4$ binding agent or an $\alpha 4$ ligand
binding agent. Preferred agents are anti-VLA4 or anti-
$\alpha 4\beta 7$ antibody homologs (human antibody, a chimeric
antibody, a humanized antibody and fragments thereof);
25 anti-VCAM-1 antibody homologs (a human antibody, a
chimeric antibody, a humanized antibody and fragments
thereof); and a small molecule inhibitor of the
interaction of $\alpha 4$ subunit-containing integrins with their
respective $\alpha 4$ integrin ligands (e.g, the VCAM-1/VLA-4
30 interaction). The composition can be administered at a
dosage so as to provide from about 0.1 to about 20 mg/kg

body weight. One or more antagonists of an interaction between an integrin with an α4 subunit and a ligand for this integrin could be administered in combination with one or more compounds that preferably are not an

5   antagonist of an interaction between an integrin with an α4 subunit and a ligand for this integrin. Preferably, the compound to be administered in combination with an antagonist of an interaction between an integrin with an α4 subunit and a ligand for this integrin is a

10   chemotherapeutic agent. Preferably, that chemotherapeutic agent is melphalan.

Unless stipulated otherwise, all references are incorporated herein by reference.

Throughout this specification and claims, the

15   word "comprise," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

20   **Figure 1.**

Effect of Neutralizing Antibodies on TRAP-positive
Multinucleated OC-like Cell Formation in the
Co-cultures of 5TGM1 cells and Bone Marrow Cells

A mixture of 5TGM1 cells (1 e 3) and marrow

25   cells (1 e 6) in suspension was plated in 48-well culture plates and cultured with or without 10 µg/ml anti-VCAM-1 antibody (VCAM-1 Ab), anti-α4beta1 antibody (α4β11Ab), anti-ICAM-1 antibody (ICAM-1 Ab) or rat IgG as a control. After 6 days of culture, cultures were fixed and the

number of TRAP-positive multinucleated OC-like cells
(TRAP(+) MNC) determined. Both VCAM-1 Ab and α4beta1 Ab
inhibited TRAP(+) MNC formation, while ICAM-1 Ab had no
effect. Data are expressed as mean ± S.E. (n=3).
* = Significantly different from IgG control.

Figure 2

Effect of 5TGM1 and ST2 Conditioned Media on Bone
Resorption in Organ Cultures of Fetal Rat Long Bones

Conditioned media (48 hours) obtained from ST2
alone, 5TGM1 alone, and co-cultures of ST2 and 5TGM1 were
assayed for bone resorbing activity in organ cultures of
^{45}calcium-labeled fetal rat long bones. Labeled fetal rat
long bones were cultured in the presence of conditioned
media (40%6v/v) or control medium for 120 hours. Data are
expressed as percentage increase of calcium release over
than in the control medium. Release from conditioned
medium of ST2 stromal cells is shown as the open bar.
Release from 5TGM1 is the hatched bar. Release from
conditioned medium harvested from co-culture of 5TGM1 and
ST2 is the closed bar. Data are expressed as mean ± S.E.
(n=4). * = significantly different from ST2 alone. *** =
significantly different from 5TGM 1 alone.

Figure 3

Effect of Recombinant Soluble VCAM-1(sVCAM-1) on the
Production of Osteoclastogenic Activity by 5TGM1 Cells

Conditioned medium was harvested from 5TGM1
cells cultured in the presence or absence of sVCAM-1
($1x10^{-8}$ to $1x10^{-7}$ Molar) for 24 hours. Osteoclastogenic
activity of these conditioned media was assayed in the

mouse marrow cultures. Bone marrow cells (1e6/well) were
plated into 48-well plates, and cultured in the presence
of conditioned media (hatched bars) or control medium
(IMDM) containing the same concentrations of sVCAM-1 (open
5   bars). After 6 days, cultures were fixed and the number
of TRAP-positive multinucleated OC-like cells (TRAP+MNC)
was determined. Conditioned medium from 5TGM1 cells
treated with 1x10^{-7} MsVCAM-1 increased TRAP(+)MNC
formation. Data are expressed as mean ± S.E. (n=3). * =
10   significantly different from controls.

Figure 4

Effect of mAb PS2 to VLA-4 on serum
IgG2b elevation in 5TGM1-bearing mice
                   Mice were injected with 1e5 5TGM1 cells, which
15   were allowed to colonize the bone marrow. Mice were split
into two groups of three, one serving as a control group,
and the second treated on days 8, 11, 14, 17, and 20 with
80 µg mAb PS/2 (~4 mg/kg). Levels of IgG2b, the antibody
isotype produced by 5TGM1 myeloma cells, were measured
20   weekly from weeks 1 to 6. mAb treatment strongly
inhibited IgG2b production, indicative of inhibition of
myeloma cell survival and growth in vivo.

Figure 5

Effect of mAb M/K-2.7 to VCAM-1 on
25   serum IgG2b elevation in 5TGM1-bearing mice
                   Mice were injected with 5TGM1 cells as described
in Figure 4, which were allowed to colonize the bone
marrow. Mice were split into groups of four or five, one
group serving as a control group (open square), the

second/third groups treated prophylactically at 80 µg (open diamonds) and 160 µg mAb (open circles) (~4 to 8 mg/kg), the fourth group treated therapeutically at 160 µg mAb (triangles). Levels of IgG2b, the antibody
5 isotype produced by 5TGM1 myeloma cells, were measured. mAb treatment strongly inhibited IgG2b production, indicative of inhibition of myeloma cell survival and growth in vivo.

Figure 6
10 Effect of anti-α4 Integrin Antibody
on Survival of Multiple Myeloma-bearing Mice

Figure 7 depicts an experimental protocol of an in vivo combination therapy experiment.

Figure 8: Serum IgG$_2$ Levels in 5TGM1-bearing Mice
15 Figure 8 shows the results (serum IgG2b levels in mice bearing 5TGM-1 cells) of an in vivo combination therapy experiment.
Ctr= Control. Mel= melphalan. α4Ab = anti-α4Ab.

Figure 9: Tumor Volume in Bone
20 Figure 9 shows the results (tumor volume in bone) of an in vivo combination therapy experiment.
Ctr= Control. Mel= melphalan. α4Ab = anti-α4Ab.

Figure 10: Effect of PS/2 mAb Treatment on Spleen Weight
Figure 10 shows the results (spleen weight) of an
25 experiment relating to PS/2 mAb administration in an in

vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

Figure 11: **Effect of PS/2 Treatment on Splenocytes in Mice with 5TGM1 Myeloma**

5   Figure 11 shows the results (percentage of myeloma cells in the spleen) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

10   **Figure 12:** **Effect of PS/2 mAb Treatment on Splenocyte FACS Profile**

Figure 12 shows the representative FACS plots of the staining for lineage markers and cytoplasmic mouse IgG2b in Splenocytes of an experiment relating to PS/2 mAb

15   administration in an in vivo model system for multiple myeloma. Four panels are shown: disease free; untreated; PS/2 treated and rat IgG2b treated. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

Figure 13: **Effect of PS/2 mAb Treatment on Spleen**

20   **Tumor Burden**

Figure 13 shows the results (spleen tumor burden) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

25   **Figure 14:** **Effect of PS/2 mAb Treatment on Bone Marrow Cells in Mice with 5TGM1 Myeloma**

Figure 14 shows the results (effects on bone marrow cells) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

Figure 15:

Effect of PS/2 mAb Treatment on Bone Marrow Tumor Burden mIgG2b Positive Cells

Figure 15 shows the results (bone marrow tumor burden) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

Figure 16:   Effect of PS/2 mAb Treatment on 5TGM1 Cells in the Blood

Figure 16 shows the results (effects on 5TGM1 myeloma cells in peripheral blood) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

Figure 17A; Figure 17B and Figure 17C:

Effect of PS/2 Treatment on Blood Chemistry

Figures 17A, 17B and 17C show the results (effects on blood chemistry: LDH levels (Fig. 17A); AST levels (Fig. 17B) and phosphorus levels (Fig. 17C)) of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

LDH = lactate dehydrogenase. AST = aspartate
aminotransferase.

Figure 18:   **Effect of PS/2 mAb on Circulation mIgG2b**
                    Levels

5   Figure 18 shows the results (effects on circulating mIgG2b
    levels at the conclusion of the experiment) of an
    experiment relating to PS/2 mAb administration in an in
    vivo model system for multiple myeloma. The mice of this
    model system were immuno-competent C57BL/KaLwRij mice.

10 **Figure 19:**

    C57BL/KaLwRij Multiple Myeloma Model

    Acute Treatment Protocols

    Figure 19 depicts an experimental acute treatment protocol
    of an experiment relating to PS/2 mAb administration in an
15 in vivo model system for multiple myeloma. The mice of
    this model system were immuno-competent C57BL/KaLwRij
    mice.

    Figure 20A and Figure 20B:

    Effect of PS/2 Treatment on IgG2b Positive Cells

20 **Acute Treatment Model**

    Figure 20A and 20B show the results (effects on IgG2b
    positive cells; 1 day treatment (**Figure 20A**) and 2 day
    treatment (**Figure 20B**)) of an acute treatment protocol of
    an experiment relating to PS/2 mAb administration in an in
25 vivo model system for multiple myeloma. The mice of this
    model system were immuno-competent C57BL/KaLwRij mice.

Figure 21: **Effect of Anti-VLA4-Antibody PS/2 on Tumor Burden in Spleen**

Figure 21 shows the results (effects on tumor burden in spleen; 1 and 2 day treatments) of an acute treatment

5   protocol of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. Three panels are shown. The panels are, from left to right, total cells, IgG2b negative cells and IgG2b positive cells. The mice of this model system were

10   immuno-competent C57BL/KaLwRij mice.

Figure 22: **Effect of 6 Days of PS/2 Treatment**

Figure 22 shows the results (effects on IgG2b positive cells; 6 day treatments) of an acute treatment protocol of an experiment relating to PS/2 mAb administration in an in

15   vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

Figure 23: **Effect of 6 Days of PS/2 Treatment on Bone Marrow cells**

Figure 23 shows the results (effects on tumor burden in

20   bone marrow; 6 day treatments) of an acute treatment protocol of an experiment relating to PS/2 mAb administration in an in vivo model system for multiple myeloma. The mice of this model system were immuno-competent C57BL/KaLwRij mice.

25   BM cells = bone marrow cells.

Figure 24:   C57BL/KaLwRij Model of Multiple Myeloma
                     Combination Treatment with PS/2 and
                     Melphalan

Figure 24 depicts an experimental protocol of an in vivo
combination therapy experiment in immunocompetent
C57BL/KaLwRij mice.
rIgG2b isotype control = rat IGg2b isotype control.

Figure 25:   **Effect of Treatment on mIgG2b Plasma Levels**

Figure 25 shows the results that melphalan and PS/2 mAb
together, but not either alone, reduced mIgG2b levels in
an in vivo combination therapy experiment in
immunocompetent C57BL/KaLwRij mice.
rIgG2b = rat IGg2b.

Figures 26A, 26B and 26C: Effect of Treatment on Myeloma
Cells at Day 27

Figures 26A, 26B and 26C show the results (effects of
treatment on myeloma cells at day 27: in blood (Fig. 26A);
in spleen (Fig. 26B) and in bone marrow (Fig. 26C)) of an
in vivo combination therapy experiment in immunocompetent
C57BL/KaLwRij mice.

Figures 27A and 27B:
Effect of Treatment on Tumor Burden at Day 27

Figures 27A and 27B shows the results (effects of
treatment on tumor burden at day 27: in spleen (Figure
27A) and in bone marrow (Figure 27B)) of an in vivo
combination therapy experiment in immunocompetent
C57BL/KaLwRij mice.

Figure 28 shows PS/2 mAb plasma levels of an in vivo combination therapy experiment in immunocompetent C57BL/KaLwRij mice.

5 **Figure 29:**
C57BL/KaLwRij Model of Multiple Myeloma
PS/2 and Anti-VLA4 Small Molecule
Long Term Treatment
Figure 29 depicts an experimental protocol of an
10 experiment relating to BIO8809 administration in an in vivo model system (immunocompetent C57BL/KaLwRij mouse model) for multiple myeloma.
8809 = BIO8809. 9257 = BIO9257.

Figures 30A, 30B and 30C:
15 **Effect of Long Term Treatment on Myeloma Cells**
Figures 30A, 30B and 30C show the results (effects of long term treatment on myeloma cells: in blood (Figure 30A), in spleen (Figure 30B) and in bone marrow (Figure 30C)) of an experiment relating to BIO8809 administration in an in
20 vivo model system (immunocompetent C57BL/KaLwRij mouse model) for multiple myeloma.
8809 = BIO8809. 9257 = BIO9257.

Figures 31A, 31B and 31C:
Effect of Long Term Treatment on Tumor Burden
25 Figures 31A, 31B and 31C show the results (effects of long term treatment on tumor burden: in blood (Fig. 31A); in spleen (Fig. 31B) and in bone marrow (Fig. 31C)) of an

experiment relating to BIO8809 administration in an in vivo model system (immunocompetent C57BL/KaLwRij mouse model) for multiple myeloma.

8809 = BIO8809. 9257 = BIO9257.

5 **Figure 32:**

C57BL/KaLwRij Model of Multiple Myeloma

PS/2 and Anti-VLA4 Small Molecule

Acute Treatment

Figure 32 depicts an acute treatment protocol of an
10 experiment relating to BIO8809 administration in an in vivo model system (immunocompetent C57BL/KaLwRij mouse model) for multiple myeloma.

8809 = BIO8809. 9257 = BIO9257.

Figures 33A, 33B and 33C:
15 **Effect of Acute Treatment on Myeloma Cells**

Figures 33A, 33B and 33C show the results (effects of acute treatment on myeloma cells: in blood (Fig. 33A); in spleen (Fig. 33B) and in bone marrow (Fig. 33C)) of an experiment relating to BIO8809 administration in an in
20 vivo model system (immunocompetent C57BL/KaLwRij mouse model) for multiple myeloma.

8809 = BIO8809. 9257 = BIO9257.

Figures 34A and 34B:

Effect of Acute Treatment on Tumor Burden

25 Figures 34A and 34B show the results (effects of acute term treatment on tumor burden: in spleen (Fig. 34A) and in bone marrow (Fig. 34B)) of an experiment relating to

BIO8809 administration in an in vivo model system
(immunocompetent C57BL/KaLwRij mouse model) for multiple
myeloma.
8809 = BIO8809.   9257 = BIO9257.

5   **Figure 35** depicts an experimental protocol of an in vivo
combination therapy experiment relating to survival as
measured by the onset of hindleg paralysis.

Figure 36: Inhibition of Hindleg Paralysis
shows the results of an in vivo combination therapy
10 experiment relating to survival as measured by the onset
of hindleg paralysis.
Mel = melphalan.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to treatments for, among
15 other things, preventing multiple myeloma. More
particularly, methods of the invention relate to the use
of antagonists of an interaction between an integrin
containing an $\alpha 4$ subunit and a ligand for this integrin
in the treatment of multiple myeloma. The term "multiple
20 myeloma" is intended to mean a medical condition in an
individual having a neoplastic disease of plasma cells,
with the neoplastic clone representing cells at different
stages in the plasma cell lineage from patient to patient
(Mundy, 1998).
25   Alpha 4 $\beta 1$ integrin is a cell-surface receptor
for VCAM-1, fibronectin and possibly other molecules that
bind with, or otherwise interact with, alpha 4 $\beta 1$
integrin. In this regard, such molecules that bind with,

or otherwise interact with, alpha 4 subunit containing integrin are individually and collectively referred to as "α4 ligand(s)"). The term α4β1 integrin ("VLA-4" or "α4β1, or "α4β1 integrin", used interchangeably) herein

5   thus refers to polypeptides which are capable of binding to VCAM-1 and members of the extracellular matrix proteins, most particularly fibronectin, or homologs or fragments thereof, although it will be appreciated by workers of ordinary skill in the art that other ligands

10 for VLA-4 may exist and can be analyzed using conventional methods.

     Nevertheless, it is known that the α4 subunit will associate with other β subunits besides β1 so that we may define the term "α4 integrin" as being those

15 integrins whose α4 subunit associates with one or another of the β subunits. A further example of an "α4" integrin is α4β7 (R. Lobb and M. Hemler, 1994). As used herein, the term "α4 integrin(s)" means VLA-4, as well as integrins that contain β1, β7 or any other β subunit.

20   As discussed herein, the antagonists used in methods of the invention are not limited to a particular type or structure of molecule so that, for purposes of the invention, any agent capable of binding to any integrin containing an α4 subunit such as VLA-4 on the surface of

25 VLA-4 bearing cells and/or α4β7 integrin on the surface of α4β7-bearing cells [see Lobb and Hemler, J. Clin. Invest., 94: 1722-1728 (1994)] and/or to their respective α4 ligands such as VCAM-1 and MadCAM, respectively, on the surface of VCAM-1 and MadCAM bearing cells, and which

30 effectively blocks or coats VLA-4 (or α4β7) or VCAM-1 (or MadCAM) (i.e., a "an α4 integrin binding agent" and "α4

integrin ligand binding agent" respectively), is considered to be an equivalent of the antagonists used in the examples herein.

An integrin "antagonist" includes any compound that inhibits an $\alpha 4$ integrin(s) from binding with an $\alpha 4$ integrin ligand and/or receptor. Anti-integrin antibody or antibody homolog-containing proteins (discussed below) as well as other molecules such as soluble forms of the ligand proteins for integrins are useful. Soluble forms of the ligand proteins for $\alpha 4$ integrins include soluble VCAM-1 or collagen peptides, VCAM-1 fusion protein, or bifunctional VCAM-1/Ig fusion proteins. For example, a soluble form of an $\alpha 4$ integrin ligand or a fragment thereof may be administered to bind to integrin, and preferably compete for an integrin binding site on cells, thereby leading to effects similar to the administration of antagonists such as anti-$\alpha 4$ integrin (e.g., $\alpha 4\beta 7$ antibodies and/or VLA-4 antibodies). In particular, soluble $\alpha 4$ integrin mutants that bind $\alpha 4$ integrin ligand but do not elicit integrin-dependent signaling are included within the scope of the invention. Such mutants can act as competitive inhibitors of wild type integrin protein and are considered "antagonists". Other antagonists used in the methods of the invention are "small molecules", as defined below.

Included within the invention are methods using an agent that antagonizes the action of more than one $\alpha 4$ integrin, such as a single small molecule or antibody homolog that antagonizes several $\alpha 4$ integrins such as VLA-4 and $\alpha 4\beta 7$, or other combinations of $\alpha 4$ integrins. Also included within the scope of the invention are

methods using a combination of different molecules such
that the combined activity antagonizes the action of more
than one α4 integrin, such as methods using several
small molecules or antibody homologs that in combination
5 antagonize the α4 integrins VLA-4 and α4β7, or other
combinations of integrins.

As discussed herein, certain integrin
antagonists can be fused or otherwise conjugated to, for
instance, an antibody homolog such as an immunoglobulin or
10 fragment thereof and are not limited to a particular type
or structure of an integrin or ligand or other molecule.
Thus, for purposes of the invention, any agent capable of
forming a fusion protein (as defined below) and capable of
binding to α4 integrin ligands and which effectively
15 blocks or coats α4β7 and/or VLA-4 integrin is considered
to be an equivalent of the antagonists used in the
examples herein.

For the purposes of the invention an "antagonist
of the α4 integrin ligand/α4 integrin interaction" refers
20 to an agent, e.g., a polypeptide or other molecule, which
can inhibit or block α4 ligand (e.g., VCAM-1) and/or α4
integrin (e.g., α4β7 or VLA-4)-mediated binding or which
can otherwise modulate α4 ligand and/or α4 integrin
function, e.g., by inhibiting or blocking α4-ligand-
25 mediated α4 integrin signal transduction or α4 ligand
mediated α4 ligand signal transduction and which is
effective in the treatment of multiple myeloma, preferably
in the same manner as are anti-α4 integrin antibodies.

Specifically, an antagonist of the VCAM-1/VLA-4
30 interaction is an agent which has one or more of the
following properties: (1) it coats, or binds to VLA-4 on

the surface of a VLA-4 bearing cell (e.g., a myeloma cell)
with sufficient specificity to inhibit a VLA-4-
ligand/VLA-4 interaction, e.g., the VCAM-1/VLA-4
interaction between bone stromal cells and myeloma cells;
5   (2) it coats, or binds to, VLA-4 on the surface of a VLA-4
bearing cell (i.e., a myeloma cell) with sufficient
specificity to modify, and preferably to inhibit,
transduction of a VLA-4-mediated signal e.g., VLA-
4/VCAM-1-mediated signaling; (3) it coats, or binds to, a
10   VLA-4-ligand, (e.g., VCAM-1) on bone stromal cells with
sufficient specificity to inhibit the VLA-4/VCAM
interaction; (4) it coats, or binds to, a VLA-4-ligand
(e.g., VCAM-1) on bone stromal cells with sufficient
specificity to modify, and preferably to inhibit,
15   transduction of VLA-4-ligand mediated VLA-4 signaling,
e.g., VCAM-1-mediated VLA-4 signaling. In preferred
embodiments the antagonist has one or both of properties 1
and 2. In other preferred embodiments the antagonist has
one or both of properties 3 and 4. Moreover, more than
20   one antagonist can be administered to a patient, e.g., an
agent which binds to VLA-4 can be combined with an agent
which binds to VCAM-1.

        For example, antibodies or antibody homologs
(discussed below) as well as soluble forms of the natural
25   binding proteins for VLA-4 and VCAM-1 are useful. Soluble
forms of the natural binding proteins for VLA-4 include
soluble VCAM-1 peptides, VCAM-1 fusion proteins,
bifunctional VCAM-1/Ig fusion proteins, fibronectin,
fibronectin having an alternatively spliced non-type III
30   connecting segment, and fibronectin peptides containing
the amino acid sequence EILDV or a similar conservatively

substituted amino acid sequence. Soluble forms of the natural binding proteins for VCAM-1 include soluble VLA-4 peptides, VLA-4 fusion proteins, bifunctional VLA-4/Ig fusion proteins and the like. As used herein, a "soluble

5   VLA-4 peptide" or a "soluble VCAM-1 peptide" is an VLA-4 or VCAM-1 polypeptide incapable of anchoring itself in a membrane. Such soluble polypeptides include, for example, VLA-4 and VCAM polypeptides that lack a sufficient portion of their membrane spanning domain to anchor the

10 polypeptide or are modified such that the membrane spanning domain is non-functional. These binding agents can act by competing with the cell-surface binding protein for VLA-4 or by otherwise altering VLA-4 function. For example, a soluble form of VCAM-1(see, e.g., Osborn et al.

15 1989, Cell, 59: 1203-1211) or a fragment thereof may be administered to bind to VLA-4, and preferably compete for a VLA-4 binding site on myeloma cells, thereby leading to effects similar to the administration of antagonists such as small molecules or anti-VLA-4 antibodies.

20   In another example, VCAM-1, or a fragment thereof, which is capable of binding to VLA-4 on the surface of VLA-4 bearing myeloma cells, e.g., a fragment containing the two N-terminal domains of VCAM-1, can be fused to a second peptide, e.g., a peptide which increases

25 the solubility or the in vivo life time of the VCAM-1 moiety. The second peptide can be a fragment of a soluble peptide, preferably a human peptide, more preferably a plasma protein, or a member of the immunoglobulin superfamily. In particularly preferred embodiments the

30 second peptide is IgG or a portion or fragment thereof,

e.g., the human IgG1 heavy chain constant region and includes, at least the hinge, CH2 and CH3 domains.

Other antagonists useful in the methods of the invention include, but are not limited to, agents that
5   mimic the action of peptides (organic molecules called "small molecules") capable of disrupting the α4 integrin/α4 integrin ligand interaction by, for instance, blocking VLA-4 by binding VLA-4 receptors on the surface of cells or blocking VCAM-1 by binding VCAM-1 receptors on
10 the surface of cells. These "small molecules" may themselves be small peptides, or larger peptide-containing organic compounds or non-peptidic organic, compounds. A "small molecule", as defined herein, is not intended to encompass an antibody or antibody homolog. Although the
15 molecular weight of such "small molecules" is generally less than 2000, we don't intend to apply this figure as an absolute upper limit on molecular weight.

For instance, small molecules such as oligosaccharides that mimic the binding domain of a VLA-4
20 ligand and fit the receptor domain of VLA-4 may be employed. (See, J.J. Devlin et al., 1990, Science 249: 400-406 (1990), J.K. Scott and G.P. Smith, 1990, Science 249: 386-390, and U.S. Patent 4,833,092 (Geysen), all incorporated herein by reference.) Conversely, small
25 molecules that mimic the binding domain of a VCAM-1 ligand and fit the receptor domain of VCAM-1 may be employed.

Examples of other small molecules useful in the invention can be found in Komoriya et al. ("The Minimal Essential Sequence for a Major Cell Type-Specific Adhesion
30 Site (CS1) Within the Alternatively Spliced Type III Connecting Segment Domain of Fibronectin Is
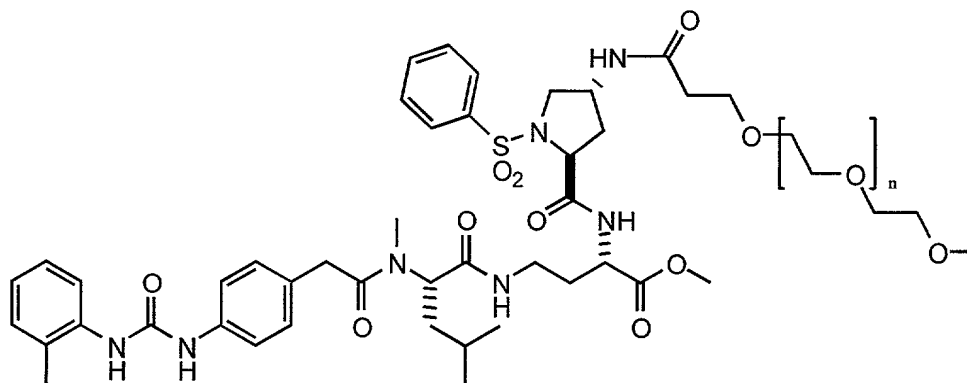
Leucine-Aspartic Acid-Valine", J. Biol. Chem., 266 (23),
pp. 15075-79 (1991)). They identified the minimum active
amino acid sequence necessary to bind VLA-4 and
synthesized a variety of overlapping peptides based on the

5   amino acid sequence of the CS-1 region (the VLA-4 binding
domain) of a particular species of fibronectin. They
identified an 8-amino acid peptide, Glu-Ile-Leu-Asp-Val-
Pro-Ser-Thr, as well as two smaller overlapping
pentapeptides, Glu-Ile-Leu-Asp-Val and

10 Leu-Asp-Val-Pro-Ser, that possessed inhibitory activity
against fibronectin-dependent cell adhesion. Certain
larger peptides containing the LDV sequence were
subsequently shown to be active in vivo (T.A. Ferguson
et al., "Two Integrin Binding Peptides Abrogate

15 T-cell-Mediated Immune Responses In Vivo", Proc. Natl.
Acad. Sci. USA, 88, pp. 8072-76 (1991); and S. M. Wahl
et al., "Synthetic Fibronectin Peptides Suppress Arthritis
in Rats by Interrupting Leukocyte Adhesion and
Recruitment", J. Clin. Invest., 94, pp. 655-62 (1994)).

20 A cyclic pentapeptide, Arg-Cys-Asp-TPro-Cys (wherein TPro
denotes 4-thioproline), which can inhibit both VLA-4 and
VLA-5 adhesion to fibronectin has also been described.
(See, e.g., D.M. Nowlin et al., "A Novel Cyclic
Pentapeptide Inhibits Alpha4Beta1 Integrin-mediated Cell

25 Adhesion", J. Biol, Chem., 268(27), pp. 20352-59 (1993);
and PCT publication PCT/US91/04862). This pentapeptide
was based on the tripeptide sequence Arg-Gly-Asp from FN
which had been known as a common motif in the recognition
site for several extracellular-matrix proteins.

30   Examples of other small molecule VLA-4
inhibitors have been reported, for example, in Adams

et al., "Cell Adhesion Inhibitors", PCT US97/13013,
describing linear peptidyl compounds containing beta-amino
acids which have cell adhesion inhibitory activity.
International patent applications WO 94/15958 and
5   WO 92/00995 describe cyclic peptide and peptidomimetic
compounds with cell adhesion inhibitory activity.
International patent applications WO 93/08823 and WO
92/08464 describe guanidinyl-, urea- and
thiourea-containing cell adhesion inhibitory compounds.
10   United States Patent No. 5,260,277 describes guanidinyl
cell adhesion modulation compounds.

Examples of small molecules that bind to or
otherwise interact with VLA-4 molecules (and/or that
specifically inhibit the binding of a ligand to VLA-4) and
15   inhibit VLA-4 dependent cell adhesion are disclosed in PCT
publication WO 01/12186 A1, published February 22, 2001
(PCT application number PCT/US00/22285, filed August 14,
2000), the disclosure of which is incorporated by
reference herein. A preferred small molecule is BIO-8809,
20   shown below:

Small molecules mimetic agents may be produced by synthesizing a plurality of peptides semi-peptidic compounds or non-peptidic, organic compounds, and then screening those compounds for their ability to inhibit the $\alpha 4$ integrin/$\alpha 4$ integrin ligand interaction. See generally U.S. Patent No. 4,833,092, Scott and Smith, "Searching for Peptide Ligands with an Epitope Library", Science, 249, pp. 386-90 (1990), and Devlin et al., "Random Peptide Libraries: A Source of Specific Protein Binding Molecules", Science, 249, pp. 40407 (1990).

In other preferred embodiments, the agent that is used in the method of the invention to bind to, including block or coat, cell-surface $\alpha 4$ integrin and/or $\alpha 4$ integrin ligand is an anti-VLA-4 and/or anti-$\alpha 4\beta 7$ monoclonal antibody or antibody homolog. Preferred antibodies and homologs for treatment, in particular for human treatment, include human antibody homologs, humanized antibody homologs, chimeric antibody homologs, Fab, Fab', F(ab')2 and F(v) antibody fragments, and monomers or dimers of antibody heavy or light chains or mixtures thereof. Monoclonal antibodies against VLA-4 are a preferred binding agent in the method of the invention.

As used herein, the term "antibody homolog" includes intact antibodies consisting of immunoglobulin light and heavy chains linked via disulfide bonds. The term "antibody homolog" is also intended to encompass a protein comprising one or more polypeptides selected from immunoglobulin light chains, immunoglobulin heavy chains and antigen-binding fragments thereof which are capable of binding to one or more antigens. The component polypeptides of an antibody homolog composed of more than

one polypeptide may optionally be disulfide-bound or otherwise covalently crosslinked.

Accordingly, therefore, "antibody homologs" include intact immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof), wherein the light chains of the immunoglobulin may be of types kappa or lambda.

"Antibody homologs" also include portions of intact antibodies that retain antigen-binding specificity, for example, Fab fragments, Fab' fragments, F(ab')2 fragments, F(v) fragments, heavy chain monomers or dimers, light chain monomers or dimers, dimers consisting of one heavy and one light chain, and the like. Thus, antigen-binding fragments, as well as full-length dimeric or trimeric polypeptides derived from the above-described antibodies are themselves useful.

As used herein, a "humanized antibody homolog" is an antibody homolog, produced by recombinant DNA technology, in which some or all of the amino acids of a human immunoglobulin light or heavy chain that are not required for antigen binding have been substituted for the corresponding amino acids from a nonhuman mammalian immunoglobulin light or heavy chain.

As used herein, a "chimeric antibody homolog" is an antibody homolog, produced by recombinant DNA technology, in which all or part of the hinge and constant regions of an immunoglobulin light chain, heavy chain, or both, have been substituted for the corresponding regions from another immunoglobulin light chain or heavy chain. In another aspect the invention features a variant of a chimeric molecule which includes: (1) a VLA-4 targeting

moiety, e.g., a VCAM-1 moiety capable of binding to antigen (i.e., VLA-4) on the surface of VLA-4 bearing myeloma cells; (2) optionally, a second peptide, e.g., one which increases solubility or in vivo life time of the VLA-4 targeting moiety, e.g., a member of the immunoglobulin superfamily or fragment or portion thereof, e.g., a portion or a fragment of IgG, e.g., the human IgG1 heavy chain constant region, e.g., CH2 and CH3 hinge regions; and a toxin moiety. The VLA-4 targeting moiety can be any naturally occurring VLA-4 ligand or fragment thereof, e.g., a VCAM-1 peptide or a similar conservatively substituted amino acid sequence. A preferred targeting moiety is a soluble VCAM-1 fragment, e.g., the N-terminal domains 1 and 2 of the VCAM-1 molecule. The chimeric molecule can be used to treat a subject, e.g., a human, at risk for disorder, e.g., multiple myeloma, characterized by the presence of myeloma cells bearing VLA-4, and preferably activated VLA-4.

As used herein, a "human antibody homolog" is an antibody homolog produced by recombinant DNA technology, in which all of the amino acids of an immunoglobulin light or heavy chain that are derived from a human source.

Methods of Making Anti-VLA-4 Antibody Homologs

The technology for producing monoclonal antibody homologs is well known. Briefly, an immortal cell line (typically myeloma cells) is fused to lymphocytes (typically splenocytes) from a mammal immunized with whole cells expressing a given antigen, e.g., VLA-4, and the culture supernatants of the resulting hybridoma cells are

screened for antibodies against the antigen. See,
generally, Kohler et al., 1975, Nature, 265: 295-297.

Immunization may be accomplished using standard
procedures. The unit dose and immunization regimen depend
on the species of mammal immunized, its immune status, the
body weight of the mammal, etc. Typically, the immunized
mammals are bled and the serum from each blood sample is
assayed for particular antibodies using appropriate
screening assays. For example, anti-VLA-4 antibodies may
be identified by immunoprecipitation of 125I-labeled cell
lysates from VLA-4-expressing cells. (See, Sanchez-Madrid
et al., 1986, Eur. J. Immunol., 16: 1343-1349 and Hemler
et al., 1987, J. Biol. Chem., 262, 11478-11485).
Anti-VLA-4 antibodies may also be identified by flow
cytometry, e.g., by measuring fluorescent staining of
Ramos cells incubated with an antibody believed to
recognize VLA-4 (see, Elices et al., 1990 Cell, 60:
577-584). The lymphocytes used in the production of
hybridoma cells typically are isolated from immunized
mammals whose sera have already tested positive for the
presence of anti-VLA-4 antibodies using such screening
assays.

Typically, the immortal cell line (e.g., a
myeloma cell line) is derived from the same mammalian
species as the lymphocytes. Preferred immortal cell lines
are mouse myeloma cell lines that are sensitive to culture
medium containing hypoxanthine, arninopterin and thymidine
("HAT medium"). Typically, HAT-sensitive mouse myeloma
cells are fused to mouse splenocytes using 1500 molecular
weight polyethylene glycol ("PEG 1500"). Hybridoma cells
resulting from the fusion are then selected using HAT

medium, which kills unfused and unproductively fused
myeloma cells (unfused splenocytes die after several days
because they are not transformed). Hybridomas producing a
desired antibody are detected by screening the hybridoma
culture supernatants. For example, hybridomas prepared to
produce anti-VLA-4 antibodies may be screened by testing
the hybridoma culture supernatant for secreted antibodies
having the ability to bind to a recombinant α4-subunit-
expressing cell line (see, Elices et al., supra).

To produce anti-VLA-4 antibody homologs that are
intact immunoglobulins, hybridoma cells that tested
positive in such screening assays were cultured in a
nutrient medium under conditions and for a time sufficient
to allow the hybridoma cells to secrete the monoclonal
antibodies into the culture medium. Tissue culture
techniques and culture media suitable for hybridoma cells
are well known. The conditioned hybridoma culture
supernatant may be collected and the anti-VLA4 antibodies
optionally further purified by well-known methods.

Alternatively, the desired antibody may be
produced by injecting the hybridoma cells into the
peritoneal cavity of an unimmunized mouse. The hybridoma
cells proliferate in the peritoneal cavity, secreting the
antibody which accumulates as ascites fluid. The antibody
may be harvested by withdrawing the ascites fluid from the
peritoneal cavity with a syringe.

Several mouse anti-VLA-4 monoclonal antibodies
have been previously described. See, e.g., Sanchez-Madrid
et al.,1986, supra; Hemler et al., 1987, supra; Pulido
et al., 1991, J. Biol. Chem., 266 (16), 10241-10245).
These anti-VLA-4 monoclonal antibodies such as HP ½ and

other anti-VLA-4 antibodies (e.g., HP2/1, HP2/4, L25,
P4C2, P4G9) capable of recognizing the P chain of VLA-4
will be useful in the methods of treatment according to
the present invention. Anti VLA-4 antibodies that will
5   recognize the VLA-4 α4 chain epitopes involved in binding
to VCAM-1 and fibronectin ligands (i.e., antibodies which
can bind to VLA-4 at a site involved in ligand recognition
and block VCAM-1 and fibronectin binding) are preferred.
Such antibodies have been defined as B epitope-specific
10   antibodies (B1 or B2) (Pulido et al., 1991, supra) and are
also anti-VLA-4 antibodies according to the present
invention.

Fully human monoclonal antibody homologs against
VLA-4 are another preferred binding agent which may block
15   or coat VLA-4 antigens in the method of the invention. In
their intact form these may be prepared using in
vitro-primed human splenocytes, as described by Boerner
et al.,1991, J. Immunol.,147, 86-95. Alternatively, they
may be prepared by repertoire cloning as described by
20   Persson et al., 1991, Proc. Nat. Acad. Sci. USA, 88:
2432-2436 or by Huang and Stollar, 1991, J. Immunol.
Methods 141, 227-236. U.S. Patent 5,798,230
(Aug. 25,1998, "Process for the preparation of human
monoclonal antibodies and their use") who describe
25   preparation of human monoclonal antibodies from human B
cells. According to this process, human
antibody-producing B cells are immortalized by infection
with an Epstein-Barr virus, or a derivative thereof, that
expresses Epstein-Barr virus nuclear antigen 2 (EBNA2).
30   EBNA2 function, which is required for immortalization, is

subsequently shut off, which results in an increase in antibody production.

In yet another method for producing fully human antibodies, United States Patent 5,789,650 (Aug. 4, 1998,
5   "Transgenic non-human animals for producing heterologous antibodies") describes transgenic non-human animals capable of producing heterologous antibodies and transgenic non-human animals having inactivated endogenous immunoglobulin genes. Endogenous immunoglobulin genes are
10 suppressed by antisense polynucleotides and/or by antiserum directed against endogenous immunoglobulins. Heterologous antibodies are encoded by immunoglobulin genes not normally found in the genome of that species of non-human animal. One or more transgenes containing
15 sequences of unrearranged heterologous human immunoglobulin heavy chains are introduced into a non-human animal thereby forming a transgenic animal capable of functionally rearranging transgenic immunoglobulin sequences and producing a repertoire of
20 antibodies of various isotypes encoded by human immunoglobulin genes. Such heterologous human antibodies are produced in B-cells which are thereafter immortalized, e.g., by fusing with an immortalizing cell line such as a myeloma or by manipulating such B-cells by other
25 techniques to perpetuate a cell line capable of producing a monoclonal heterologous, fully human antibody homolog.

Large nonimmunized human phage display libraries may also be used to isolate high affinity antibodies that can be developed as human therapeutics using standard
30 phage technology (Vaughan et al., 1996). Yet another preferred binding agent which may block or coat VLA-4

antigens in the method of the invention is a humanized
recombinant antibody homolog having anti-VLA-4
specificity. Following the early methods for the
preparation of chimeric antibodies, a new approach was

5   described in EP 0239400 (Winter et al.) whereby antibodies
are altered by substitution of their complementarity
determining regions (CDRs) for one species with those from
another. This process may be used, for example, to
substitute the CDRs from human heavy and light chain Ig

10 variable region domains with alternative CDRs from murine
variable region domains. These altered Ig variable
regions may subsequently be combined with human Ig
constant regions to created antibodies which are totally
human in composition except for the substituted murine

15 CDRs. Such CDR-substituted antibodies would be predicted
to be less likely to elicit an immune response in humans
compared to chimeric antibodies because the
CDR-substituted antibodies contain considerably less
non-human components. The process for humanizing

20 monoclonal antibodies via CDR "grafting" has been termed
"reshaping". (Riechmann et al., 1988, Nature 332,
323-327; Verhoeyen et al., 1988, Science 239, 1534-1536).

    Typically, complementarity determining regions
(CDRs) of a murine antibody are transplanted onto the

25 corresponding regions in a human antibody, since it is the
CDRs (three in antibody heavy chains, three in light
chains) that are the regions of the mouse antibody which
bind to a specific antigen. Transplantation of CDRs is
achieved by genetic engineering whereby CDR DNA sequences

30 are determined by cloning of murine heavy and light chain
variable (V) region gene segments, and are then

transferred to corresponding human V regions by site directed mutagenesis. In the final stage of the process, human constant region gene segments of the desired isotype (usually gamma I for CH and kappa for CL) are added and

5 the humanized heavy and light chain genes are co-expressed in mammalian cells to produce soluble humanized antibody.

The transfer of these CDRs to a human antibody confers on this antibody the antigen binding properties of the original murine antibody. The six CDRs in the murine

10 antibody are mounted structurally on a V region "framework" region. The reason that CDR-grafting is successful is that framework regions between mouse and human antibodies may have very similar 3-D structures with similar points of attachment for CDRs, such that CDRs can

15 be interchanged. Such humanized antibody homologs may be prepared, as exemplified in Jones et al., 1986, Nature 321, 522-525; Riechmann, 1988, Nature 332, 323-327; Queen et al.,1989, Proc. Nat. Acad. Sci. USA 86, 10029; and Orlandi et al., 1989, Proc. Nat. Acad. Sci. USA 86, 3833.

20 Nonetheless, certain amino acids within framework regions are thought to interact with CDRs and to influence overall antigen binding affinity. The direct transfer of CDRs from a murine antibody to produce a recombinant humanized antibody without any modifications

25 of the human V region frameworks often results in a partial or complete loss of binding affinity. In a number of cases, it appears to be critical to alter residues in the framework regions of the acceptor antibody in order to obtain binding activity.

30 Queen et al., 1989 (supra) and WO 90/07861 (Protein Design Labs) have described the preparation of a

humanized antibody that contains modified residues in the framework regions of the acceptor antibody by combining the CDRs of a murine mAb (anti-Tac) with human immunoglobulin framework and constant regions. They have

5   demonstrated one solution to the problem of the loss of binding affinity that often results from direct CDR transfer without any modifications of the human V region framework residues; their solution involves two key steps. First, the human V framework regions are chosen by

10   computer analysts for optimal protein sequence homology to the V region framework of the original murine antibody, in this case, the anti-Tac mAb. In the second step, the tertiary structure of the murine V region is modeled by computer in order to visualize framework amino acid

15   residues which are likely to interact with the murine CDRs and these murine amino acid residues are then superimposed on the homologous human framework. See also Protein Design Labs -- U.S. Patent 5,693,762.

One may use a different approach (Tempest

20   et al., 1991, Biotechnology 9, 266-271) and utilize, as standard, the V region frameworks derived from NEWM and REI heavy and light chains respectively for CDR-grafting without radical introduction of mouse residues. An advantage of using the Tempest et al., approach to

25   construct NEWM and REI based humanized antibodies is that the 3-dimensional structures of NEWM and REI variable regions are known from x-ray crystallography and thus specific interactions between CDRs and V region framework residues can be modeled.

30   Regardless of the approach taken, the examples of the initial humanized antibody homologs prepared to

date have shown that it is not a straightforward process.
However, even acknowledging that such framework changes
may be necessary, it is not possible to predict, on the
basis of the available prior art, which, if any, framework

5   residues will need to be altered to obtain functional
humanized recombinant antibodies of the desired
specificity. Results thus far indicate that changes
necessary to preserve specificity and/or affinity are for
the most part unique to a given antibody and cannot be

10 predicted based on the humanization of a different
antibody.

Preferred antagonists useful in the present
invention include chimeric recombinant and humanized
recombinant antibody homologs (i.e., intact

15 immunoglobulins and portions thereof) with B epitope
specificity that have been prepared and are described in
co-pending U.S. Patent Application Serial No. 08/004,798,
filed January 12, 1993, PCT Publication US94/00266, filed
January 7, 1994. The starting material for the

20 preparation of chimeric (mouse V - human C) and humanized
anti-VLA-4 antibody homologs may be a murine monoclonal
anti-VLA-4 antibody as previously described, a monoclonal
anti-VLA-4 antibody commercially available (e.g., HP2/1,
Amae International, Inc., Westbrook, Maine), or a

25 monoclonal anti-VLA-4 antibody prepared in accordance with
the teaching herein. For example, the variable regions of
the heavy and light chains of the anti-VLA-4 antibody HP ½
have been cloned, sequenced and expressed in combination
with constant regions of human immunoglobulin heavy and

30 light chains. Such HP ½ antibody is similar in
specificity and potency to the murine HP ½ antibody, and

may be useful in methods of treatment according to the present invention.

Other preferred humanized anti-VLA4 antibody homologs are described by Athena Neurosciences, Inc. in
5   PCT/US95/01219 (27 July 1995). These humanized anti-VLA-4 antibodies comprise a humanized light chain and a humanized heavy chain. The humanized light chain comprises three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the
10   corresponding complementarity determining regions of a mouse 21-6 immunoglobulin light chain, and a variable region framework from a human kappa light chain variable region framework sequence except in at least position the amino acid position is occupied by the same amino acid
15   present in the equivalent position of the mouse 21.6 immunoglobulin light chain variable region framework. The humanized heavy chain comprises three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity
20   determining regions of a mouse 21-6 immunoglobulin heavy chain, and a variable region framework from a human heavy chain variable region framework sequence except in at least one position the amino acid position is occupied by the same amino acid present in the equivalent position of
25   the mouse 21-6 immunoglobulin heavy chain variable region framework.

Therapeutic Applications

In this method according to the first aspect of the invention, VLA-4 binding agents, in particular, VCAM
30   fusions and anti-VLA-4 antibody homologs are preferably

administered parenterally. The term "parenteral" as used
herein includes subcutaneous, intravenous, intramuscular,
intra-articular, intra-synovial, intrasternal,
intrathecal, intrahepatic, intralesional and intracranial
injection or infusion techniques.

The VLA-4 binding agents are preferably
administered as a sterile pharmaceutical composition
containing a pharmaceutically acceptable carrier, which
may be any of the numerous well known carriers, such as
water, saline, phosphate buffered saline, dextrose,
glycerol, ethanol, and the like, or combinations thereof.
The compounds of the present invention may be used in the
form of pharmaceutically acceptable salts derived from
inorganic or organic acids and bases. Included among such
acid salts are the following: acetate, adipate, alginate,
aspartate, benzoate, benzenesulfonate, bisulfate,
butyrate, citrate, camphorate, camphorsulfonate,
cyclopentanepropionate, digluconate, dodecylsulfate,
ethanesulfonate, fumarate, glucoheptanoate,
glycerophosphate, hemisulfate, heptanoate, hexanoate,
hydrochloride, hydrobromide, hydroiodide,
2-hydroxyethanesulfonate, lactate, maleate,
methanesulfonate, 2-naphthalenesulfonate, nicotinate,
oxalate, pamoate, pectinate, persulfate,
3-phenyl-propionate, picrate, pivalate, propionate,
succinate, tartrate, thiocyanate, tosylate and
undecanoate. Base salts include ammonium salts, alkali
metal salts, such as sodium and potassium salts, alkaline
earth metal salts, such as calcium and magnesium salts,
salts with organic bases, such as dicyclohexylamine salts,
N-methyl-D-glucamine, tris(hydroxymethyl)methylamine and

salts with amino acids such as arginine, lysine, and so forth. Also, the basic nitrogen-containing groups can be quaternized with such agents as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides and iodides; dialkyl sulfates, such as dimethyl, diethyl, dibutyl and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides, aralkyl halides, such as benzyl and phenethyl bromides and others. Water or oil soluble or dispersible products are then obtained.

The pharmaceutical compositions of this invention comprise any of the compounds of the present invention, or pharmaceutically acceptable derivatives thereof, together with any pharmaceutically acceptable carrier. The term "carrier" as used herein includes acceptable adjuvants and vehicles. Pharmaceutically acceptable carriers that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

According to this invention, the pharmaceutical compositions may be in the form of a sterile injectable preparation, for example a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques -known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as do natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as Ph. Helv or similar alcohol.

The pharmaceutical compositions of this invention, in particular small molecule antagonists of the VLA-4/VCAM-1 interaction, may be given parenterally or orally. If given orally, they can be administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch.

Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are required for oral

5   use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added. Topically-transdermal patches may also be used. The pharmaceutical compositions of this invention may also be

10 administered by nasal aerosol or inhalation through the use of a nebulizer, a dry powder inhaler or a metered dose inhaler. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline,

15 employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional solubilizing or dispersing agents.

According to another embodiment compositions

20 containing a compound of this invention may also comprise an additional agent selected from the group consisting of corticosteroids, anti-inflammatories, immunosuppressants, antimetabolites, and immunomodulators. Specific compounds within each of these classes may be selected from any of

25 those listed under the appropriate group headings in "Comprehensive Medicinal Chemistry", Pergamon Press, Oxford, England, pp. 970-986 (1990), the disclosure of which is herein incorporated by reference. Also included within this group are compounds such as theophylline,

30 sulfasalazine and aminosalicylates (anti-inflammatories); cyclosporin, FK-506, and rapamycin (immunosuppressants);

cyclophosphamide and methotrexate (antimetabolites);
steroids (inhaled, oral or topical) and interferons
(immunomodulators).

The amount of active ingredient that may be
5   combined with the carrier materials to produce a single
dosage form will vary depending upon the host treated, and
the particular mode of administration. It should be
understood, however, that a specific dosage and treatment
regimen for any particular patient will depend upon a
10   variety of factors, including the activity of the specific
compound employed, the age, body weight, general health,
sex, diet, time of administration, rate of excretion, drug
combination, and the judgment of the treating physician
and the severity of the particular disease being treated.
15   The amount of active ingredient may also depend upon the
therapeutic or prophylactic agent, if any, with which the
ingredient is co-administered.

The dosage and dose rate of the compounds of
this invention effective to prevent, suppress or inhibit
20   cell adhesion will depend on a variety of factors, such as
the nature of the inhibitor, the size of the patient, the
goal of the treatment, the nature of the pathology to be
treated, the specific pharmaceutical composition used, and
the judgment of the treating physician. Dosage levels of
25   between about 0.001 and about 100 mg/kg body weight per
day, preferably between about 0.1 and about 50 mg/kg body
weight per day of the active ingredient compound are
useful. Most preferably, the VLA-4 binding agent, if an
antibody or antibody derivative, will be administered at a
30   dose ranging between about 0.1 mg/kg body weight/day and
about 20 mg/kg body weight/day, preferably ranging between

about 0.1 mg/kg body weight/day and about 10 mg/kg body
weight/day and at intervals of every 1-14 days. For
non-antibody or small molecule binding agents, the dose
range should preferably be between molar equivalent

5   amounts to these amounts of antibody. Preferably, an
antibody composition is administered in an amount
effective to provide a plasma level of antibody of at
least 1 mg/ml. Optimization of dosages can be determined
by administration of the binding agents, followed by

10 assessment of the coating of VLA-4-positive cells by the
agent over time after administered at a given dose in
vivo.

        Myeloma cells contained in a sample of the
individual's peripheral blood (or bone marrow cells)

15 should be probed for the presence of the agent in vitro
(or ex vivo) using a second reagent to detect the
administered agent. For example, this may be a
fluorochrome labeled antibody specific for the
administered agent which is then measured by standard FACS

20 (fluorescence activated cell sorter) analysis.
Alternatively, presence of the administered agent may be
detected in vitro (or ex vivo) by the inability or
decreased ability of the individual's cells to bind the
same agent which has been itself labeled (e.g., by a

25 fluorochrome). The preferred dosage should produce
detectable coating of the vast majority of VLA-4-positive
cells. Preferably, coating is sustained in the case of an
antibody homolog for a 1-14 day period.

Combination Therapy

In some embodiments of this invention, one or more antagonists of an interaction between an integrin with an α4 subunit and a ligand for this integrin could be administered in combination with one or more compounds

5 that may not be (preferably are not) an antagonist of an interaction between an integrin with an α4 subunit and a ligand for this integrin. Such compound could be a chemotherapeutic agent or another agent, including without limitation: melphalan, bisphosphonates (examples of which

10 are ibandronate and pamidronate), thalidomide, erythropoietin, and antagonists, such as mAb blockers, of IL6 and IL15.

Multiple myeloma is currently treated inefficiently with standard chemotherapeutic regimens. In

15 some embodiments of this invention, an antagonist of an interaction between an integrin with an α4 subunit and a ligand for this integrin could be administered in combination with one or more standard agents for treatment of multiple myeloma, such as chemotherapeutic agents.

20 Hence, the two compounds (an antagonist of an interaction between an integrin with an α4 subunit and a ligand for this integrin and a compound that may not be (preferably is not) an antagonist of an interaction between an integrin with an α4 subunit and a ligand for this

25 integrin; said compound that may not be (preferably is not) an antagonist of an interaction between an integrin with an α4 subunit and a ligand for this integrin could be a chemotherapeutic agent or another agent for treatment or prevention of multiple myeloma, including, inter alia:

30 melphalan, bisphosphonates (examples of which are ibandronate and pamidronate), thalidomide, erythropoietin,

and antagonists, such as mAb blockers, of IL6 and IL15) could act to prevent or treat multiple myeloma synergistically; or lower dosage of either or both compounds (an antagonist of an interaction between an

5 integrin with an α4 subunit and a ligand for this integrin and a compound that may not be (preferably is not) an antagonist of an interaction between an integrin with an α4 subunit and a ligand for this integrin, such as one or more standard agents for treatment of multiple

10 myeloma) needed to provide the same effect as a higher dosage of either compound alone.

In some embodiments, this invention provides methods for treating or preventing multiple myeloma comprising administering to an individual a

15 therapeutically or prophylactically effective amount of a first composition comprising an antagonist of an interaction between an α4 subunit-bearing integrin and a ligand for an α4 subunit-bearing integrin, wherein said first composition is administered in combination with a

20 therapeutically or prophylactically effective amount of a second composition comprising a compound that may not be (preferably is not) an antagonist of an interaction between an α4 subunit-bearing integrin and a ligand for an α4 subunit-bearing integrin. In certain embodiments,

25 this invention provides methods for treating or preventing multiple myeloma comprising administering to an individual a therapeutically or prophylactically effective amount of a first composition comprising an antagonist of an interaction between an α4 subunit-bearing integrin and a

30 ligand for an α4 subunit-bearing integrin, wherein said first composition is administered in combination with a

therapeutically or prophylactically effective amount of a
second composition comprising a compound that may not be
(preferably is not) an antagonist of an interaction
between an α4 subunit-bearing integrin and a ligand for

5   an α4 subunit-bearing integrin, wherein, to be
therapeutically or prophylactically effective, a dosage of
said antagonist is lower when administered in combination
with said compound than not administered in combination
with said compound; or

10 a dosage of said compound is lower when administered in
combination with said antagonist than not administered in
combination with said antagonist,
or both. Such compound could be a chemotherapeutic agent
or another agent for treating or preventing multiple

15 myeloma, including, for example: melphalan,
bisphosphonates (examples of which are ibandronate and
pamidronate), thalidomide, erythropoietin, and
antagonists, such as mAb blockers, of IL6 and IL15.

        In some embodiments, this invention provides

20 methods for inhibiting bone resorption associated with
tumors of bone marrow, the methods comprising
administering to a mammal with said tumors an antagonist
of an interaction between an α4 subunit-bearing integrin
and a ligand for an α4 subunit-bearing integrin, in an

25 amount effective to provide inhibition of said bone
resorption, wherein said antagonist is administered in
combination with a compound, in an amount effective to
provide inhibition of said bone resorption, that may not
be (preferably is not) an antagonist of an interaction

30 between an α4 subunit-bearing integrin and a ligand for
an α4 subunit-bearing integrin. In certain embodiments,

this invention provides methods for inhibiting bone
resorption associated with tumors of bone marrow, the
methods comprising administering to a mammal with said
tumors an antagonist of an interaction between an $\alpha 4$

5   subunit-bearing integrin and a ligand for an $\alpha 4$ subunit-
bearing integrin, in an amount effective to provide
inhibition of said bone resorption, wherein said
antagonist is administered in combination with a compound,
in an amount effective to provide inhibition of said bone

10   resorption, that may not be (preferably is not) an
antagonist of an interaction between an $\alpha 4$
subunit-bearing integrin and a ligand for an $\alpha 4$ subunit-
bearing integrin, wherein, to be therapeutically or
prophylactically effective,

15   a dosage of said antagonist is lower when administered in
combination with said compound than not administered in
combination with said compound; or
a dosage of said compound is lower when administered in
combination with said antagonist than not administered in

20   combination with said antagonist,
or both. Such compound could be a chemotherapeutic agent
or another agent for inhibiting bone resorption associated
with tumors of bone marrow, including, for example:
melphalan, bisphosphonates (examples of which are

25   ibandronate and pamidronate), thalidomide, erythropoietin,
and antagonists, such as mAb blockers, of IL6 and IL15.
       In some embodiments, this invention provides
methods of treating or preventing a subject having a
disorder characterized by the presence of

30   osteoclastogenesis, the methods comprising administering
to the subject an antagonist of an interaction between an

α4 subunit-bearing integrin and a ligand for an α4
subunit bearing integrin, in an amount sufficient to
suppress or prevent the osteoclastogenesis, wherein said
antagonist is administered in combination with a compound,

5 in an amount sufficient to suppress or prevent the
osteoclastogenesis, that may not be (preferably is not) an
antagonist of an interaction between an α4
subunit-bearing integrin and a ligand for an α4 subunit-
bearing integrin. In certain embodiments, this invention

10 provides methods of treating or preventing a subject
having a disorder characterized by the presence of
osteoclastogenesis, the methods comprising administering
to the subject an antagonist of an interaction between an
α4 subunit-bearing integrin and a ligand for an α4

15 subunit bearing integrin, in an amount sufficient to
suppress or prevent the osteoclastogenesis, wherein said
antagonist is administered in combination with a
compound, in an amount sufficient to suppress or prevent
the osteoclastogenesis, that may not be (preferably is

20 not) an antagonist of an interaction between an α4
subunit-bearing integrin and a ligand for an α4 subunit-
bearing integrin, wherein, to be therapeutically or
prophylactically effective,
a dosage of said antagonist is lower when administered in

25 combination with said compound than not administered in
combination with said compound; or
a dosage of said compound is lower when administered in
combination with said antagonist than not administered in
combination with said antagonist,

30 or both. Such compound could be a chemotherapeutic agent
or another agent for treating or preventing a subject

having a disorder characterized by the presence of
osteoclastogenesis, including, for example: melphalan,
bisphosphonates (examples of which are ibandronate and
pamidronate), thalidomide, erythropoietin, and

5   antagonists, such as mAb blockers, of IL6 and IL15.

         In some embodiments, the pharmaceutical or
prophylactic composition of this invention can also
include a pharmaceutically or prophylactically effective
amount of a chemotherapeutic agent or another agent,

10   including without limitation: melphalan, bisphosphonates
(examples of which are ibandronate and pamidronate),
thalidomide, erythropoietin, and antagonists, such as mAb
blockers, of IL6 and IL15. Said chemotherapeutic agent or
another agent could be included in a second composition.

15   Animal Models

         The animal model has been described in detail
(Garrett 1997). Briefly, Radl et al. (1988) had described
a murine model of myeloma which arose spontaneously in
aged C57BL/KaLwRij mice. This condition occurred in

20   approximately 1 in 200 animals as they aged, and led to a
monoclonal gammopathy with some of the features of human
disease (Radl 1988). To develop a better and more
reproducible animal model we have established and
subcloned a cell line from this murine myeloma called

25   5TGM1, and found that it causes lesions in mice
characteristic of human myeloma, such as severe osteolysis
and the involvement of non-bone organs including liver and
kidney (Garrett 1997). Mice inoculated with cultured
cells develop disease in a highly predictable and

30   reproducible manner, which includes formation of a

monoclonal gammopathy and radiologic bone lesions. Furthermore, some of the mice become hypercalcemic, and the bone lesions are characterized by increased osteoclast activity. Thus, based on histological examination of affected organs in 5TGM1-bearing mice and increased serum levels of IgG2b, 5TGM1 is defined as a murine myeloma which recapitulates accurately the hallmarks of human disease.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature. In the following examples, the necessary restriction enzymes, plasmids, and other reagents and materials may be obtained from commercial sources and cloning, ligation and other recombinant DNA methodology may be performed by procedures well-known in the art.

Example 1: MATERIALS AND METHODS

5TGM1 Myeloma Cells

5TGM1 myeloma cells were initially derived from a myeloma which arose spontaneously in aged C57BL/KaLwRij mice (Garrett 1997, Vanderkerken 1997). Cells were grown in Isocove's Modified Dulbecco's Medium (IMDM, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Summit, Fort Collins, CO) and 1% penicillin-streptomycin solution (GIBCO, Grand Island, NY) at 37 C in 5% CO_2 atmosphere. For in vitro experimentation described below, 5TGM1 cells between passage 25 and 30 were used.

Antibodies, soluble VCAM-1

Neutralizing antibodies against murine VCAM-1 (M/K-2.7), integrin VLA-4 (PS/2 mAb), and Intercellular Adhesion Molecule-1 (ICAM-1, YN1/1.7), were kindly gifted
5 by Dr. Kensuke Miyake (Saga Medical University, Saga, Japan). Recombinant soluble VCAM-1 (Lobb et al., 1991), containing the 7 extracellular domains of human VCAM-1, was the gift of Dr. Roy Lobb, Biogen Inc., Cambridge, MA.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
10 Using RT-PCR, we confirmed expression of VCAM-1 and integrin $\alpha 4$ in bone marrow stromal cells and 5TGM1, respectively. Total RNA was prepared from 5TGM1, a primary culture of bone marrow stromal cells and an ST2 marrow stromal cell line (RIKEN Cell Bank, Tsukuba, Japan)
15 by the single-step RNA isolation method using TRIzol reagent (GIBCO). Three μg of RNA was incubated with 50 ng of random hexamer at 70° C for 10 min and chilled on ice, then converted to first strand cDNA using reverse transcriptase (Perkin-Elmer, Branchburg, NJ) according to
20 the manufacturers instruction. The primers used for PCR were as follows: murine VCAM-1 5'-primer; 5'-OH-GCTGCGCGTCACCATTGTTCTC-3'-OH (SEQ ID NO: 1]; murine VCAM-1 3'-primer, 5'-OH-ACCACCCTCTTGAAGCCTTGTG-3'-OH [SEQ NO: 2]; murine integrin $\alpha 4$ 5'-primer, 5'-OH-
25 CCCCTCAACACGAACAGATAGG-3'-OH [SEQ ID NO: 3]; murine integrin $\alpha 4$ 3'-primer; 5'-OH-GCCTTGTCCTTAGCAACACTGC-3'-OH [SEQ ID NO:4].

PCR was performed for 30 cycles consisting of 1 min at 94° C,1 min at 55° C and 2 min at 72° C. PCR
30 reaction mixture (total 50 μl) contained 10 microliters.

First strand cDNA, 50 mM KCL, 10 mM Tris-HCL (pH 8.3), 2 mM MgCl$_2$, deoxy-NTP mix (0.2 mM each), the pair of primers (0.15 micromolar each) and 2 U Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ). The PCR products were

5   separated on 2.5% agarose gels containing ethidium bromide and visualized under ultraviolet light. The size of the fragments were confirmed by reference to molecular weight markers.

Attachment of 5TGM1 Cells onto Bone Marrow Stromal Cells

10   For heterotypic cell-cell adhesion assays, ST2 cells (5 e 4/well) were seeded in 48-well culture plates (Costar, Cambridge, MA) and cultured 48 h in alphaMEM supplemented with 10% FBS until confluency. 5TGM1 cells (5 e 6) were labeled by incubation with 10 microCi

15   [methyl-3H] thymidine (New England Nuclear) for 24 h at 37° C in the culture medium. After the ST2 monolayer was formed, it was incubated with 1% bovine serum albumin (BSA, Sigma, St Louis, MO) in serum-free alphaMEM for 1 hour and tritium-labeled 5TGM1 cells were plated onto the

20   monolayer. The system was incubated in the absence or presence of antibodies to VCAM-1 or $\alpha 4\beta 1$ integrin at 37° C for 1 h. Non-adherent cells were removed by washing with 5% trichloroacetic acid twice and PBS twice, and adherent cells were solubilized in 300 microliters of 0.25

25   mM NaOH, neutralized with the same volume of 0.25 mm HCl and the radioactivity was determined in a liquid scintillation counter.

Osteoclast Formation Assay in the Co-culture of 5TGM1 and Mouse Bone Marrow Cells

Mouse bone marrow cells were obtained from 5-week-old male C57BL mice as described previously (Yoneda 1993). Femurs and tibiae were dissected aseptically and both ends cut off. Bone marrow cells were flushed out, collected and incubated in alphaMEM supplemented with 10% FBS (Hyclone, Logan, UT) and 1% penicillin-streptomycin in 100 mm-culture dishes (Becton Dickinson Labware, Bedford, MA) at 37°C for 2 h. Non-adherent cells containing hemopoietic osteoclast precursors and stromal cells were harvested. Bone marrow cells (1 e 6) and 5TGM1 cells (1 e 3) in 300 microliters of the culture medium were plated onto 48-well culture plates (day 0). On day 2, 300 microliters of fresh culture medium was gently added to each well, and on day 4, 300 microliters of spent medium was replaced with the same volume of fresh medium. On day 6, the cultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using commercial kits (Sigma). TRAP-positive multinucleated cells with more than 3 nuclei were defined as osteoclast-like (OC-like) cells, and manually counted under microscope. To confirm that these OC-like cells have the capability to resorb bone, 5TGM1 cells and marrow cells were co-cultured on 5x5 mm whale dentine slices in the same condition, and resorption pits formed on these dentine slices were examined by scanning electron microscopy as described (Yoneda 1992).

In some experiments, co-cultures of 5TGM1 myeloma cells and marrow cells were performed using transwell inserts (Becton Dickinson Ubware) to prevent

direct contact between these two types of cells. (2 e 6, 24-well plates, Costar). Marrow cells were plated in the lower chambers and 5TGM1 myeloma cells (2 e 3) were then plated in either lower (direct contact) or upper (no

5   contact) chambers.

Organ Cultures of 45Ca-labeled Fetal Rat Long Bones

Conditioned media harvested from 5TGM1 cultures were assayed for bone-resorbing activity by organ cultures of 45Ca-labeled fetal rat long bones as described

10 previously (Mbalaviele 1995). Pregnant rats were injected with 250 uCi of 45Ca (New England Nuclear) on the 18th day of gestation. Radius and ulna bone shafts were obtained from 19-day fetuses by microdissection, and precultured for 24 h in BGJ medium (Sigma) supplemented with 0.1% BSA

15 between air and liquid-phase on stainless mesh grids. Bones were then cultured in the presence of conditioned media (50% v/v) or in control medium for 120 hours. The media were changed once at 48 hours. At the end of the culture, bones were incubated in ice-cold 5%

20 trichloroacetic acid for 2h, and 45Ca radioactivity in bones and media determined in a liquid scintillation counter. Bone resorption was quantitated as the percentage of 45Ca released into the medium from bones as calculated by: (45Ca count in medium)/ (45Ca count in

25 medium and bone) x 100.

Co-culture of 5TGM1 Myeloma Cells with Mouse Stromal Cell Line ST2 Cells

ST2 cells (0.5 e 6) and 5TGM1(4 e 6) cells were plated together onto 60-mm culture dishes (Becton Dickinson) in 10% FBS-supplemented IMDM and cultured overnight, washed with serum-free IMDM twice, and incubated in 5 ml of serum-free IMDM. After 48 h, conditioned media were harvested and stored at -70° C until use.

Effect of mAb PS2 to VLA-4 on serum IgG2b elevation in 5TGM1-bearing mice

Mice were injected with 1 e 5 5TGM1 cells, which were allowed to colonize the bone marrow. Mice were split into two groups of three, one serving as a control group, and the second treated biweekly beginning on day 8 with 80 µg mAb PS/2 (4 mg/kg). Levels of IgG2b, the antibody isotype produced by 5TGM1 myeloma cells, were measured weekly from weeks 1 to 6.

RESULTS

Expression of VCAM-1, VLA-4, and effect of Antibodies Against VCAM-1 and VLA-4 on 5TGM1 Attachment to ST2 Monolayers

Using RT-PCR, we confirmed the expression of VCAM-1 and integrin VLA-4 in bone marrow stromal cells and myeloma cells, respectively. As expected, both the ST2 stromal cell line and primary bone marrow stromal cells expressed VCAM-1, while 5TGM1 did not. In contrast, the 5TGM1 myeloma cells expressed integrin VLA-4, whereas stromal cells did not (data not shown). In addition, both

anti-VCAM-1 antibody (10 µg/ml) and VLA-4 antibody (10 µg/ml) partially (50-80%) inhibited the attachment of 5TGM1 cells to ST2 monolayers, showing that VCAM-1 and the VLA-4 integrin expressed on these cells are biologically

5   functional and that these antibodies have neutralizing activity (data not shown).

OC-like Cell Formation in the Coculture of 5TGM1 Myeloma Cells with Mouse Bone Marrow Cells

On day 6 of the coculture of 5TGM1 cells and

10   mouse marrow cells, numerous TRAP-positive multinucleated osteoclast-like (OC-like) cells were formed. These OC-like cells exhibited resorption pit formation on dentine slices, demonstrating that these cells were capable of resorbing bone, and possess an osteoclastic phenotype. In

15   experiments using transwell inserts, formation of OC-like cells was observed when 5TGM1 cells were cultured in direct contact with bone marrow cells. In contrast, there was only a marginal number of OC-like cells formed when 5TGM1 cells were separated from marrow cells by the

20   transwell membrane. Thus 5TGM1 cells induce osteoclast formation in mixed marrow cultures, and this induction requires direct cell-cell contact.

Effect of Antibodies Against VCAM-1 and Integrin VLA4 on OC-like Cell Formation in the Co-culture of 5TGM1 and

25   **Marrow Cells**

Both anti-VCAM-1 antibody (VCAM-1 Ab, 10 µg/ml) and anti VLA-4-integrin antibody ($\alpha 4\beta 1$ Ab, 10 µg/ml) dramatically inhibited OC-like cell formation. In

contrast, mAb against ICAM-1, another adhesion molecule on marrow stromal cells implicated in stromal/myeloma interactions, had no effect on OC-like cell formation (Figure 1).

5   To determine whether this inhibition by VCAM-1 and VLA-4 mAbs was specific for 5TGM1-induced OC-like cell formation and was not due to cytotoxicity, the effects of these antibodies were examined on OC-like cell formation induced by 1,25 $(OH)_2D_3$, a widely-used stimulator of

10   osteoclastogenesis in mouse bone marrow cell cultures (Takahashi 1988). Neither VCAM-1 Ab nor VLA-4 mAb inhibited OC-like cell formation induced by vitamin D3, which itself had no effect on VCAM-1 expression in stromal cells (data not shown).

15   **Effect of conditioned Medium Harvested from the Co-culture of 5TGM1 and ST2 on Bone Resorption**

       Conditioned medium from the co-culture of 5TGM1 cells and ST2 cells showed a marked increase in bone resorption in the fetal rat long bone assay (Figure 2),

20   while conditioned medium of 5TGM1 caused only a marginal increase, as compared to control medium. Conditioned medium from ST2 cells showed no increase in bone resorption. Thus direct cell-cell contact via VCAM-1 and VLA-4 both induces osteoclast-like cells and production of

25   bone-resorbing factors in vitro.

Effect of Recombinant Soluble VCAM-1(sVCAM-1) on the Production of Bone-resorbing and Osteoclastogenic Activity by 5TGM1 Cells

Conditioned medium of 5TGM1 treated with a soluble recombinant form of VCAM-1(sVCAM-1) increased bone resorption in fetal rat long bones in a dose-dependent manner, while conditioned medium obtained from untreated

5 5TGM1 only marginally increased bone resorption. Soluble VCAM-1 itself had no effects on bone resorption (data not shown). In the mouse marrow culture system, conditioned medium harvested from 5TGM1 cells treated with sVCAM-1 showed increased activity of OC-like cell formation, while

10 conditioned medium of untreated 5TGM1 exhibited only marginal activity of OC-like cell formation (Figure 3).

Expression of Rank ligand mRNA in marrow stromal cells (ST2) cultured in the presence and absence of murine myeloma cells

15 Because Rank ligand appears to be an important mediator of OCL formation and may be the final common pathway for the effects of osteoclastogenic cytokines on OCL formation, we have examined the expression of Rank ligand in 5TGM1 and ST2 cells both individually and when

20 cocultured. We find that coculture of 5TGM1 and ST2 cells induces Rank ligand mRNA in the ST2 cells. Furthermore, while 5TGM1 cells do not express Rank ligand, they do so when treated with sVCAM-1 (not shown). Finally, the conditioned medium from 5TGM1 cells treated with sVCAM-1

25 induced Rank ligand mRNA in ST2 cells, suggesting that the VCAM-1/VLA-4 pathway produces a cytokine in myeloma cells that enhances Rank ligand expression by marrow stromal cells (data not shown).

In summary, we show that 5TGM1 cells alone
produce marginal amount of activity that stimulates
OC-like cell formation and bone resorption. However, when
5TGM1 myeloma cells were co-cultured with bone marrow

5 cells containing hemopoietic osteoclast precursors and
stromal cells, they strongly adhered to the stromal cells
and increased OC-like cell formation. There were no
OC-like cells formed in the co-cultures in which 5TGM1
cells were prevented from contacting stromal cells.

10 Furthermore, in organ cultures of fetal rat long bones the
conditioned medium harvested from the cocultures of 5TGM1
myeloma cells and ST2 bone marrow stromal cells had
increased bone resorbing activity compared with
conditioned medium of either ST2 or 5TGM1 alone. These

15 data are consistent with the notion that direct cell-cell
contact of 5TGM1 cells with bone marrow stromal cells is
required for the production of osteoclast-stimulating and
bone-resorbing activity. We then determined what cell
adhesion molecules were involved in the direct cell-cell

20 interaction between 5TGM1 cells and marrow stromal cells
that is necessary for the production of osteoclastogenic
activity. Our data indicate that VCAM-1 and VLA-4
integrin play a role in this cell-cell interaction, since
neutralizing antibodies to these two adhesion molecules

25 profoundly decreased OC-like cell formation in the
co-cultures. The VCAM-1/VLA-4 integrin interaction is
responsible for the cell-cell communication between marrow
stromal cells and 5TGM1 myeloma cells leading to increased
production of a osteoclastogenic and bone-resorbing

30 activity. Finally, this bone resorbing activity in part
is due to induction of Rank ligand.

Example 2: IN VIVO EXPERIMENTS

Our in vitro studies suggest that the interaction between VLA-4 on myeloma cells with VCAM-1 on marrow stromal cells may play a key role in the induction
5 of bone resorbing activity by myeloma. We have taken the key step of testing this hypothesis in vivo in an animal model which accurately reflects human disease.

A. In this experiment, mice were injected with 1 e 5 5TGM1 myeloma cells, which were allowed to colonize
10 the bone marrow. Mice were split into two groups of three, one serving as a control group, and the second treated biweekly beginning on day 8 with mAb PS/2. Levels of IgG2b, the antibody isotype produced by 5TGM1 myeloma cells, were measured weekly from weeks 1 to 6. Treatment
15 with mAb at a dose of 80 μg per injection (~4 mg/kg) biweekly strongly inhibited IgG2b production, indicative of significant inhibition of myeloma cell survival and growth in vivo (Figure 4). Further, the treated mice showed reduced incidence of paraplegia (all 3 untreated
20 animals showed paraplegia on day 42, while only one of the treated animals showed paraplegia). The two treated animals with no paraplegia also showed a reduction in spleen and liver weights, which also correlate with tumor burden. Finally, the treated animals showed a reduction
25 in tumor area by histology (from 6.71 +/- 1.74 to 0.05 +/- 0.08 square millimeters) in the tibia and femurs. There was no effect of treatment on serum calcium levels (data not shown)

B. In a parallel experiment, treatment with 40 μg PS/2 mAb biweekly had no effect on IgG2b levels (not shown). These data show that mAb PS/2 to VLA-4 strongly inhibits the growth of established myeloma cells in a

5   dose-dependent fashion.

C. In another in vivo experiment, 18 SCID mice were injected with 5TGM1 myeloma cells at day 0. Four mice were treated with PBS; 4 mice were treated in a prophylactic protocol with mAb M/K 2.7 reactive against to

10 mouse VCAM1 at a dosage of 80 μg (-4 mg/kg) every 3 days starting at day -1 (i.e. days - 1, 2, 5, 8, and 11). In a parallel experiment using the same protocol, five mice were treated with 160 μg mAb M/K 2.7. In addition, five mice were treated with 160 μg mAb M/K 2.7 starting at day

15 8 (i.e. days 8, 11, 14, 17, and 20) in a therapeutic protocol. Serum was taken from all mice on days 21, 28, and 35, and animals were X-rayed then sacrificed for histology on day 35. All three treatment groups showed a reduction in serum IgG2b levels, indicative of reduced

20 myeloma cell burden (Figure 5). A significant effect was also observed on spleen weights at the low dose prophylactic protocol relative to control (0.23 +/- 0.14 g for control versus 0.08 +/- 0.04 for treated). In the prophylactic high dose group 4 of 5 animals showed a clear

25 reduction in spleen weight, but the overall value was not significant because of one animal with a large spleen weight (data not presented).

D. One can investigate whether an initial high bolus dose of α4 integrin antagonist, followed by a

maintenance dose, improves efficacy. The myeloma cells are already established in the marrow compartment, and their tight VLA-4-dependent interaction with VCAM-1 needs to be inhibited. Furthermore, presumably the greater the

5   number of established myeloma cells, the higher the initial dose required to flush cells out into the peripheral circulation.

A larger study with the anti-VLA-4 antibody PS/2 (PS/2 = PS2) was therefore performed. Twenty eight SCID

10 mice were injected with 5TGM1 myeloma cells at day 0. Nine mice received no treatment; 9 mice received an isotype-matched control IgG mAb; 10 mice were treated with mAb PS/2 to alpha 4 integrin. A different therapeutic regimen was given, in which mice were given a high dose of

15 mAb (200 μg) on days 4, 5, and 6, then a maintenance dose of 80 μg (-4 mg/kg) every 3 days starting at day 8.

There was a statistically significant reduction in serum IgG2b when the treated group was compared to either the untreated or control IgG-treated group at weeks

20 3 and 4 (data not presented). Importantly, when the treated group was compared to either the untreated or control IgG-treated group there was a clear effect on survival (Figure 6).

Example 3: OTHER IN VIVO EXPERIMENTS

25   Based on the information presented herein for the first time, persons having ordinary skill in the art can readily confirm and extend the importance of the α4 integrins and their ligands in multiple myeloma using the murine animal model described.

The following series of experiments are well within the level of skill in the art based upon the present disclosure but serve merely to exemplify, and not limit, the types of work.

5 1) Dose response to mAb PS/2 to determine the optimal biweekly maintenance dose. 80 µg shows good efficacy, but 40 µg was without effect. One examines higher doses up to 20 mg/kg two or three times weekly to determine optimal dosing.

10 2) Patients present with disease at different stages of severity, linked to increased tumor burden. One examines the efficacy of mAb PS/2 given at different times after establishment of disease, i.e., one compares treatment initiation at 8 days (see for

15 example Figure 4) to initiation after two, three, four and five weeks post inoculation to see how late mAb can be given to provide sonic relief of symptoms.

3) The effects of mAb MK-2 to murine VCAM-1 are examined, following the same parameters outlined

20 above (dosing, timing of dosing) for mAb to VLA-4. It is anticipated that similar dosing levels will be required to see efficacy.

4) Further markers of myeloma progression are examined, including tumor burden in both marrow and

25 extramedullary sites, quantification of bone lesions by radiographic analysis of the skeleton by histomorphometry; measurement of rates of bone reportion by evaluation of collagen crosslinks in plasma; measurement of monoclonal protein production

in plasma; hypercalcemia where present; and
mortality.

5) Multiple myeloma is currently treated inefficiently
with standard chemotherapeutic regimens. The
additive or synergistic effects of mAbs at optimal
dosing in conjunction with, or either before or
after, dosing with appropriate chemotherapeutic
regimens is examined.

6) The ability of a small molecule $\alpha 4$ integrin
inhibitor that is selective for one particular $\alpha 4$
integrin or is selective for several $\alpha 4$ integrins at
once or the ability of combinations of such
inhibitors, to mimic the effects of mAbs and block
myeloma progression is examined using the protocols
and outcomes described above. Small molecule
inhibitors are delivered parenterally or orally, in
the dosing range of 0.1 to 30 mg/kg, once or twice
daily, or twice or three times weekly.

Example 4: **ANTI-$\alpha 4$ INTEGRIN ANTIBODY ENHANCED
SENSITIVITY OF MYELOMA CELLS TO MELPHALAN
(CHEMOTHERAPY COMBINATION EXPERIMENTS)**

Cell-cell contact of myeloma cells with stromal
cells via $\alpha 4 \beta 1$ integrin and VCAM-1 apparently facilitate
their arrest, proliferation, survival and production of
osteoclast activating factors in bone marrow cavity.
Therefore, interference with stromal cell/myeloma cell
interactions is a potential adjuvant intervention to
enhance the efficacy of anti-cancer agents in myeloma bone
disease.

Here, we studied the effects of a neutralizing
antibody to α4 integrin (α4Ab), which disrupts stromal
cells/myeloma cell interactions, on the sensitivity of
myeloma to melphalan, a most widely-used chemotherapeutic
5   agent for myeloma, using the 5TGM1 mouse myeloma cells
that reproducibly cause extensive osteolysis in tumor-
bearing animals. The α4Ab (100-200 μg/mouse, i.p., 2 or
3 times a week) and melphalan (50, 100, 200 μg/mouse,
i.p., once a week) were administered following inoculation
10   of 5TGM1 cells in the tail vein in male xid-nu-bg mice.
Melphalan alone at doses of 50 and 100 μg/mouse failed to
suppress serum IgG2 levels, a systemic indicator of
myeloma tumor burden, whereas serum IgG2 levels were
significantly suppressed by 200 μg/mouse melphalan.
15   Combined treatment with melphalan (50 or 100 μg/mouse)
and α4Ab suppressed serum IgG2 levels to a greater extent
than melphalan (200 μg/mouse) alone. Moreover,
histomorphometric examination revealed that melphalan (50
μg/mouse) combined with α4Ab significantly decreased
20   5TGM1 tumor volume in bone compared with melphalan (200
μg/mouse) alone.

         To study the role of cell-cell contact of 5TGM1
cells with marrow stromal cells in the sensitivity to
melphalan, the effects of melphalan on 5TGM1 cells
25   cultured in contact with the ST2 mouse marrow stromal
cells were examined. We found that 5TGM1 cells cultured
on ST2 cells exhibited increased survival and reduced
apoptosis in the presence of melphalan compared with 5TGM1
cells cultured on tissue culture plates. Furthermore,
30   α4Ab reduced survival and increased apoptosis in 5TGM1
cells in these cultures. In summary, our data show that

α4Ab enhanced the sensitivity of 5TGM1 cells to melphalan and suggested that disruption of stromal cell/myeloma cell interactions using α4Ab is an effective adjuvant therapy, allowing dosage reduction of chemotherapeutic agents and
5　thereby lowering the risk of adverse effects.

In one particular set of experiments on the combined effects of melphalan and anti-alpha 4 integrin Ab in 5TGM1 model, as shown in an experimental protocol in Figure 7, an initial high dose of 200 μg mAb was given
10　during the first week, followed by a maintenance dose of 100 μg. Melphalan was given three times. Animals (scid/nu/bg mice) were sacrificed at day 28 and serum IgG2b levels (a surrogate marker of tumor burden) and tumor burden in bone were measured (histomorphometric
15　analysis of bones). The serum IgG2b was only modestly affected by either melphalan or anti-α4 mAb, while the combination produced a significant drop in IgG2b levels (Fig. 8). The tumor volume in bone marrow, measured as a ratio of treated to untreated (so that a reduction results
20　in <100%), was also reduced (Fig. 9). The data were a summary of two experiments combined.

We also examined the effects of the combined administration of melphalan (100 μg/mouse) and PS/2 mAb on survival of 5TGM1-bearing mice (scid/nu/bg mice). An
25　experimental protocol of these experiments is shown in Figure 35. We evaluated survival by the onset of hindleg paralysis (i.e., the onset of hindleg paralysis was the end-point in these experiments). As shown in Figure 36, melphalan (100 μg/mouse) alone had no effect on the onset
30　of hindleg paralysis and anti-α4Ab slightly but significantly delayed the onset of hindleg paralysis.

Combined administration of melphalan and anti-α4Ab
significantly delayed the onset of hind-leg paralysis.
Kaplan-Meier analysis of these data demonstrated that the
effects of anti-α4Ab and the combination of anti-α4Ab and
5   melphalan were significantly different from the untreated.
Melphalan alone, at a dose of 200 μg/mouse, significantly
delayed the onset. Thus, we can reduce the dose of
Melphalan to a quarter in the presence of the anti-α4Ab,
as compared to melphalan alone. These data suggested that
10   combined use of the anti-α4Ab enables the reduction of
the dose of melphalan, thereby decreasing adverse effects.
        We also wish to study the dose-response of
melphalan with a fixed dose of an anti-α4 mAb.

Example 5:   **IN VIVO EXPERIMENTS CONDUCTED IN**
15   **IMMUNO-COMPETENT C57BL/KaLwRij MICE**
        Results reported below indicated a beneficial
therapeutic effect of treatment with the PS/2 mAb in a
mouse model of myeloma when initiated on day four after
infusion of myeloma cells. Mice were treated with
20   PS/2 mAb periodically and were sacrificed for analysis on
day 20 and 21.
        These experiments showed an effect of the PS/2
mAb on a number of disease parameters treatment relative
to untreated controls:

25   1.   Splenomegaly was reduced by 24% and tumor burden in
           the spleen was reduced by 75%. A disease dependent
           increase in non-myeloma cells was not affected by
           PS/2 mAb treatment.
     2.   Tumor burden in the bone marrow was reduced by 22%.

3.   Myeloma cells in the peripheral blood was reduced by 57%.

4.   Increased LDH levels were reduced by 34% and AST levels were reduced by 42%. Phosphorus levels, which decrease slightly with disease, improved by 13%.

5.   Circulating mIgG2b levels were reduced by 50% .

At the conclusion of the experiment, circulating levels of PS/2 mAb were undetectable in most mice. This indicated a possible reduction in the effectiveness of mAb treatment, probably either due to an immune response to the rat IgG or the antibody were bound up by the expanding myeloma population.

Methods

C57BL/KaLwRij mice were inoculated, via the tail vein, with 1e6 5TGM1 myeloma cells. The mice were randomly divided into three groups of ten animals each. One group was left untreated, one group received PS/2 mAb treatment and the third group received rat IgG2b isotype control mAb. The antibody treated groups received 200 micrograms of antibody on days 4,5,and 6. After that the mice were injected twice weekly with 200 micrograms of antibody. The mice were sacrificed for analysis on days 20 and 21. Blood was removed via cardiac puncture for FACS analysis of cell populations. The plasma was used for quantification of circulating mIgG2b, PS/2 mAb and numerous blood chemistry parameters. The spleen was removed and the wet weight recorded. A portion of the spleen was fixed in 4% paraformaldehyde for histological evaluation while the remainder of the spleen was used for

FACS analysis of cell populations. The liver was also removed and fixed for histological evaluation. Both femur/tibia pairs were removed. One set was fixed in 4% paraformaldehyde for histological evaluation. The bone marrow was flushed from the other pair, the cells were counted and the cell populations were analyzed by FACS.

For FACS analysis of cell populations, the cells were surface stained with a cocktail of lineage markers, which included CD4, CD8, CD19, B220, MAC1, GR1 and NK1.1. The cells were then double stained for cytoplasmic mIgG2b. Another set was surface stained for B220 alone and double stained for cytoplasmic mIgG2b.

Tumor burden was calculated by quantifying the total number of cells in either the spleen or the bone marrow (one femur/tibia pair) of each mouse. That number is then multiplied by the percentage of myeloma cells, measured as mIgG2b positive cells. The percentage of myeloma cells could also be measured as mIgG2b positive cells and/or GFP+ cells if the 5TGM1 cells are transfected with an expression vector containing the GFP gene and therefore the cells express GFP (green fluorescent protein).

The levels of mIgG2b were determined by ELISA. Plasma was incubated on plates coated with an anti mouse IgG2b antibody. The trapped mIgG2b (mouse IgG2b) was then detected with labeled anti mouse Fc.

Circulating PS/2 mAb levels were determined by a FACS binding assay. 5TGM1 cells, grown in vitro, were incubated with plasma from treated animals. The bound antibody was then detected with labeled anti rat Fc antibody.

Results

Figure 10 shows the spleen weights of each group. Spleen weight increased from an average of 89 milligrams in the disease free group to 298 milligrams in the untreated group. In the group that received PS/2 mAb treatment, the spleens were 24% smaller than the untreated group, while the control rat IgG2b isotype mAb control group was only 6% smaller. Statistical analysis using the Student t test showed that the effect of PS/2 mAb treatment was significant ($p<.05$) when compared to either the untreated or the control rat IgG2b (rIgG2b) isotype mAb control treated group. The same analysis showed that the small reduction in the control rIgG2b isotype mAb control group was not significant as compared to the untreated group.

Figure 11 shows that PS/2 mAb treatment reduced the percentage of myeloma cells in the spleen by 68%, as defined by the presence of cytoplasmic mIgG2b positive cells. This reduction seen in the PS/2 mAb treatment group was significant ($p<.001$) when compared to either the untreated or the rat IgG2b isotype mAb control groups. Figure 11 also shows that the mIgG2b positive cells were almost equally split between two populations. One population was lineage positive and the other was lineage negative. The effect of PS/2 mAb treatment is equally effective in either the lineage positive mIgG2b positive or lineage negative mIgG2b positive populations. The graph also shows that the lineage positive population was positive for the B cell marker B220. As shown in Figure 11, there was a disease dependent increase in the percentage of non myeloma cells that was not affected by

PS/2 mAb treatment. These cells were most likely an expanding extramedullary hematopoietic cell population due to the physiological stress of bone marrow neoplasia.

Figure 12 shows representative FACS plots of the staining for lineage markers and cytoplasmic mIgG2b in splenocytes. The reduction in mIgG2b positive cells in the PS/2 treated group was clearly shown.

By using the tumor burden calculation to assess the effect of PS/2 mAb treatment in the spleen, the reduction was 75%. The effect of PS/2 mAb treatment was significant (p <.001) when compared to either control group. These results are shown in Figure 13.

Figure 14 shows that, at this point in disease development, 77% of bone marrow cells were now mIgG2b positive myeloma cells. PS/2 mAb treatment resulted in a modest 15% reduction in the percentage of cytoplasmic mIgG2b positive cells in the bone marrow while there was a 6% reduction in the rat IgG2b isotype mAb control group. Analysis by Student t test showed that the reduction of mIgG2b positive cells in the PS/2 mAb treatment group was significant (p<.001) when compared to untreated group or the rat IgG2b isotype mAb control group (p<.05). As in the spleen, the mIgG2b positive population was split between a lineage positive and a lineage negative population and the effect of PS/2 mAb was on both populations. Figure 14 also shows a disease dependent depletion in the percentage of non-myeloma cells (defined as mIgG2b negative) in the bone marrow of all treatment groups. A higher percentage of bone marrow cells were retained in PS/2 mAb groups as compared to the untreated or rat IgG2b isotype mAb control groups. The PS/2 mAb

treatment was significant when compared to the untreated control group (p<.001) and the rat IgG2b control group (p<.05).

Figure 15 shows the results of the tumor burden
5 calculations for the bone marrow. These results showed that there was a 22% reduction in the absolute number of mIgG2b positive cells in the PS/2 treated group and no effect of the rat IgG2b mAb isotype control. The effect of PS/2 mAb was significant (p<.05) when compared to
10 either the untreated or rat IgG2b control groups.

The Experimental Pathology Report of the H+E stained sections indicated that there were distinctly fewer neoplastic infiltrates within the liver and spleen of PS/2 mAb treated animals as compared to either the
15 untreated or rat IgG2b control groups. In the bone marrow, no distinctly apparent differences were noted among the groups.

Figure 16 shows that mIgG2b positive myeloma cells were detected in the peripheral blood and PS/2 mAb
20 treatment reduces their numbers. The 57% reduction in mIgG2b positive cells with PS/2 mAb treatment was significant (p<.001) when compared to either the untreated or the rat IgG2b control groups.

Figures 17A, 17B and 17C show the results of the
25 blood chemistry study. As previously observed, there was an increase in LDH (Fig. 17A) and AST (Fig. 17B) levels associated with myeloma. PS/2 mAb treatment significantly reduced the levels of both enzymes as shown by a Student t test p value <.001. There was no effect of the isotype
30 control rat IgG2b antibody on these enzymes. Mice with myeloma also had a slight decrease in phosphorus levels

(Fig. 17C). PS/2 mAb treatment but not isotype control rat IgG2b mAb reduced this decrease (Fig. 17C). The PS/2 mAb effect on the phosphorus levels was significant with a Student t test p value of <.05

5   Circulating mIgG2b levels were measured by ELISA. Figure 18 shows that at the conclusion of the experiment the untreated group had an average of 2.4 mg/ml of circulating antibody while the PS/2 mAb group had a 50% reduction to 1.2 mg/ml. There was a small reduction in

10 the control rat IgG2b group to 2.0 mg/ml. The reduction in the PS/2 mAb group was significant with a Student t test p value of <.05 as compared to either the untreated or rat IgG2b control groups. The reduction in the rIgG2b control group was not significant.

15   Results of the FACS binding assay, to determine circulating PS/2 mAb levels, showed that only two of the ten animals that received PS/2 mAb treatment had detectable levels of PS/2 mAb in the plasma. This result indicated that the amount of circulating PS/2 mAb was

20 limited. This suggested that the PS/2 mAb was either fully bound to the myeloma cells or that the antibody was cleared by an immune response.

    In summary, administration of PS/2 mAb in this mouse model system of multiple myeloma had the following

25 effects: splenomegaly was reduced 24%, tumor burden in spleen by 75%, tumor burden in marrow by 22% (Fig. 15), circulating myeloma cells by 57% (Fig. 16, left hand panel [mIgG2b (pos) cells are the myeloma cells]), reduced liver enzyme levels were normalized, suggesting a reduction in

30 liver tumor burden, and circulating plasma levels of IgG2b were reduced 50%.

To evaluate the effects of mAb treatment at later times, three 'acute' treatment protocols were tried (Fig. 19). Mice were injected with 5TGM1 cells, and then given mAb PS/2 on day 16 (1 day treatment), days 16 and 17 (2 day treatment), or days 14-19 (6 day treatment).

Remarkably, as shown in Figures 20A and 20B, the percentage of IgG2b-positive (i.e. myeloma) cells was dramatically reduced in spleen and blood with either 1 (Fig. 20A) or 2 day (Fig. 20B) treatments. The tumor burden (i.e. the absolute number of myeloma cells, rather than the % of total cells) was also reduced by both treatments (Fig. 21). The 1 or 2 day treatments had no effect on cells in bone marrow, however, whether expressed as % (Figs. 20A and 20B) or tumor burden (not shown). The six day protocol reduced cells in both blood and spleen, as expected (Fig. 22). The percentage of cells in bone marrow is unaffected, but there was a clear effect when expressed as tumor burden (Fig. 23) because the total cell number and myeloma cell numbers were reduced in proportion.

These experiments are key because they suggest that: 1) very late therapeutic intervention can be successful; 2) the therapeutic effect of the anti-α4 mAb in the longer term experiments may be lost over time, due to the intact immune response to the rat antibody in the C57BL/KaLwRij mice; 3) the rapid drop in myeloma cell numbers in blood and spleen is likely not due to decreased growth but through effects on other parameters, such as apoptosis.

Chemotherapy combination experiments

Additive/synergistic effects of the combination of PS/2 mAb and melphalan were seen under most conditions. In these experiments, the 5TGM1 cells injected into the animals were transfected with an expression vector containing the GFP gene. These myeloma cells expressed the GFP protein, allowing the myeloma cells to be tracked directly as GFP+ myeloma cells. These myeloma cells could also be tracked as mIgG2b positive cells. The data were only useful on day 27. Figure 24 shows a general experimental protocol. Figure 25 shows that mIgG2b levels were unaffected by PS/2 mAb or melphalan alone, but reduced (p<0.05) by the combination. Figures 26A and 26B show that the percentages of GFP+ myeloma cells in blood (Fig. 26A) and spleen (Fig. 26B) were unaffected by melphalan but reduced by PS/2 mAb. The combination further reduced levels in spleen (Fig. 26B), but was not statistically significant in blood (Fig. 26A) because PS/2 mAb alone was so effective. Neither melphalan nor PS/2 mAb alone affected percentage of GFP+ myeloma cells in bone marrow (Fig. 26C), but the combination was effective (p<0.05). If expressed as tumor burden, the combination was once again statistically significantly better than monotherapy in both spleen (Figure 27A) and bone marrow (Figure 27B). Figure 28 shows in tabular forms that PS/2 mAb plasma levels are undetectable in all but one assay.

Example 6:   IN VIVO EXPERIMENTS CONDUCTED IN IMMUNO-COMPETENT C57BL/KaLwRij MICE: VLA4 SMALL MOLECULE EXPERIMENTS

To evaluate the efficacy of VLA4 binding small molecules, BIO8809, and a related but non-VLA4 binding chemical control, BIO9257, were used. These are PEGylated small molecules with extended half lives. BIO8809 was described in PCT publication WO 01/12186 A1, published February 22, 2001, the disclosure of which is hereby incorporated by reference. The chemical syntheses and the structures of BIO8809 and BIO9257 are shown in Example 7.

In these experiments, the 5TGM1 cells injected into the animals were transfected with an expression vector containing the GFP gene. These myeloma cells expressed the GFP protein, allowing the myeloma cells to be tracked directly as GFP+ myeloma cells. These myeloma cells could also be tracked as mIgG2b positive cells.

Tumor burden was calculated by quantifying the total number of cells in blood, the spleen or the bone marrow (one femur/tibia pair) of each mouse. That number is then multiplied by the percentage of myeloma cells, measured as GFP+ myeloma cells, to yield the tumor burden.

In two experiments, BIO8809 showed statistically significant inhibition of myeloma cell numbers in both blood and spleen, but not in bone marrow.

In a long-term treatment experiment (the protocol is summarized in Figure 29), PS/2 mAb had the expected effect on the percentage of myeloma cells in blood (Fig. 30A) and spleen (Fig. 30B), and surprisingly, in this experiment, on bone marrow as well (Fig. 30C). The small molecule had statistically significant effects in spleen (at all doses) (Fig. 30B), and in blood (0.03 mg/kg dose only) (Fig. 30A), but not in bone marrow (Fig. 30C) at any dose. Replotting as tumor burden (i.e. the

absolute number of myeloma cells) did not affect the data (Figs. 31A (blood), 31B (spleen) and 31C (bone marrow)).

In an acute 6 day treatment experiment (see protocol summarized in Figure 32; 3 day treatment for small molecules), PS/2 mAb had the expected effect on the percentage of myeloma cells in blood (Fig. 33A) and spleen (Fig. 33B). In this experiment, the untreated control in bone marrow was very low, making these data uninterpretable (Fig. 33C). The small molecule had statistically significant effects in spleen (at all doses, with nice dose response) (Fig. 33B), and in blood (all doses versus BIO9257 control; 3 mg/kg dose only versus untreated control) (Fig. 33A). In bone marrow no dose showed significant inhibition, even versus BIO9257 control (Fig. 33C). Replotting as tumor burden did not affect the data (Figs. 34A (spleen) and 34B (bone marrow)).

The small numbers (N=2 per dose) in these preliminary experiments precluded more robust analysis or interpretation. Nevertheless, these data already show that the small molecule inhibitors can work as effectively as the mAb in reducing blood and spleen myeloma burden.

Example 7: **BIO-8809 AND BIO-9257**

(2S,4R)-4-amino-1-[benzyloxycarbonyl]pyrrolidine-2-methylcarboxylate hydrochloride (**I**) could be converted to the 4-*tert*-butoxycarbonyl derivative **II** by protection of the 4-amino group with di-*tert*-butyldicarbonate and subsequent removal of the 1-benzyloxycarbonyl group using hydrogenation over Pd/C catalyst. Reaction with

benzenesulfonyl chloride gave sulfonamide **III**. Hydrolysis
of the methyl ester with lithium hydroxide gave the acid,
which was coupled to amine salt **IV** using
[O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium
5   hexafluorophosphate] (HATU) to give **V**. This methyl ester
was hydrolyzed with lithium hydroxide, followed by the
deprotection of the *tert*-butoxycarbonyl protected 4-amino
group using acidic conditions to give **VI**. This amino acid
was then coupled to methoxy-poly(ethylene
10   glycol)-succinimidyl propionate (MW: 20,000) to give
BIO-8809 (**VII**).

BIO-8809 (**VII**)
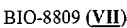
(2S,4R)-4-amino-1-[benzyloxycarbonyl]pyrrolidine-2-methylcarboxylate hydrochloride (**I**) was coupled to 6-(*tert*-butoxycarbonylamino)caproic acid using HATU to
5   give **VIII**. The benzyloxycarbonyl protecting group was hydrogenated over Pd/C catalyst, and this free amine was then coupled to benzenesulfonyl chloride to give **IX**. The methyl ester was hydrolyzed with lithium hydroxide, followed by the deprotection of the *tert*-butoxycarbonyl
10   protected 6-amino group using acidic conditions to give **X**. This amino acid was then coupled to methoxy-poly(ethylene glycol)-succinimidyl propionate (MW: 20,000) to give BIO-9257 (**XI**).

BIO-9257
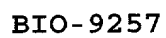
Example 8:   **OTHER COMPOUNDS**

Bisphosphonates are now standard of care in myeloma treatment (see, e.g., Berenson et al., <u>J Clin Oncol</u> 16: 593-602 (1998)), and are effective at reducing

5 bone lesions, but not tumor burden, in a particular model (see, e.g., Dallas et al., <u>Blood</u> 93: 1697-1706 (1999)). Since all myeloma patients are now on bisphosphonates, the efficacy of an VLA4 mAb or a small molecule, such as BIO8809, in combination with bisphosphonates will be

10 tried.

Thalidomide is the latest drug with reported efficacy in myeloma patients (Barlogie et al., <u>Blood</u> 98: 492-4 (2001)). Other drug candidates include erythropoietin, and mAb blockers (or other antagonists) of

15 IL6 and IL15 (Mittelman et al., <u>Proc Natl Acad Sci USA</u>, 98: 5181-5186 (2001); Tinhofer et al., <u>Blood</u> 95: 610-618 (2000); Bataille et al., 1989). The efficacy of an anti-VLA4 mAb or a small molecule, such as BIO8809, in combination with any one of these compounds, as well as

20 with melphalan, could be tried.

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